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CONTENTS

Marthella J. Frantz and Arthur Kirschbaum. Sex Hormone Secretion by Tumors of the Adrenal Cortex of Mice 257

Elizabeth Ufford Green. On a Special Condition of the Interphase Nucleus in Normal and Cancerous Cells 267

Carroll A. Pfeiffer. Development of Leiomyomas in Female Rats with an Endocrine Imbalance 277

Willemina M. de Bruyn, Remmert Korteweg, and E. Kits van Waveren. Transplantable Mouse Lymphosarcoma T 86157 (MB) Studied *in vivo, in vitro* and at Autopsy 282

A. Plaut and H. Sobel. Human Sebum as Vehicle for Methylcholanthrene 294

G. A. LePage. Effects of Phosphate Bond Energy on Nucleic Acid Stability in Tumor Homogenates 297

C. C. Lushbaugh and P. E. Steiner. Intraocular Transplantation of Malignant Lymphomas of the Mouse, Dog, and Man in Heterologous Species 299

Max S. Dunn, Evelyn R. Feaver, and Edward A. Murphy. The Amino Acid Composition of a Fibrosarcoma and Its Normal Homologous Tissue in the Rat 306

M. M. Black, I. S. Kleiner, and H. Bolker. Glycolytic Enzyme Inhibitor Therapy in Human Malignant Neoplasia 314

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CANCER RESEARCH

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Sex Hormone Secretion by Tumors of the Adrenal Cortex of Mice*

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Tumors of the adrenal cortex may be induced by ovariectomy or castration in certain strains of mice (4, 15). In the NH stock such tumors appear spontaneously, primarily in females (3). It has been demonstrated that cortical tumors of mice secrete estrogenic hormone (4, 7, 15), and that androgenic hormone may also be produced (16). The purpose of this report is to present the results of a comparative study on the secretory activity of cortical tumors arising in the Bagg albino, NH, CBA, C3H, and Strong A strains.

MATERIALS AND METHODS

Ten Bagg albino males were castrated at weaning age and were killed for study 2 years later. Eight untreated control animals of the same age and sex were similarly studied. Four males were examined 6 months after removal of the testes at weaning age. Serial sections of the adrenal glands were cut at 4 microns following fixation in Zenker's fluid. One set of sections was stained with Delafield's hematoxylin and eosin, alternate sections with Delafield's hematoxylin and basic fuchsin, the latter dye being used to detect the presence of "ceroid" pigment (2, 9, 12). Sections of the seminal vesicles, kidneys, and submaxillary glands were studied for evidence of androgenic secretion. The gross appearance of the seminal vesicles was observed. Testes from 4 Bagg albino males 1 year of age, and from 8 males 2 or more years of age were studied histologically. The sections were studied as indicated above for the adrenals.

Thirteen females of the same stock were ovariectomized at weaning age, and their vaginal smears were followed for evidence of estrogenic stimulation by secretion of adrenal cortical tumors. Four of these animals were killed at 5 and 9 at 10 months after operation. Seminal vesicles of weanling Bagg albino mice were transplanted subcutaneously into the axillae of 2 of these females whose vaginae had failed to show signs of estrogenic stimulation. The purpose was to demonstrate growth and development of the seminal vesicles under the influence of androgen liberated from the adrenal tumors induced by ovariectomy. The uteri and vaginae of these mice were sectioned longitudinally for study.

Fifty-seven male mice 4 to 6 weeks of age of the NH strain were castrated. Six or more months following operation these animals were autopsied. Seminal vesicles, kidneys, and submaxillary glands were studied for the histologic alterations induced in these organs by sex hormone secretions. Fifteen females of the same stock were ovariectomized at weaning age, and autopsied 6 months later. Here again the adrenals, submaxillary glands, kidneys, and reproductive tracts were studied histologically. Six females of the CBA, 6 of the C3H, and 3 of the Strong A strain were ovariectomized and autopsied for study 12 to 16 months later. Seminal vesicles of weanling mice of the appropriate strains were transplanted subcutaneously into the axillae of the ovariectomized females 2 months before autopsy.

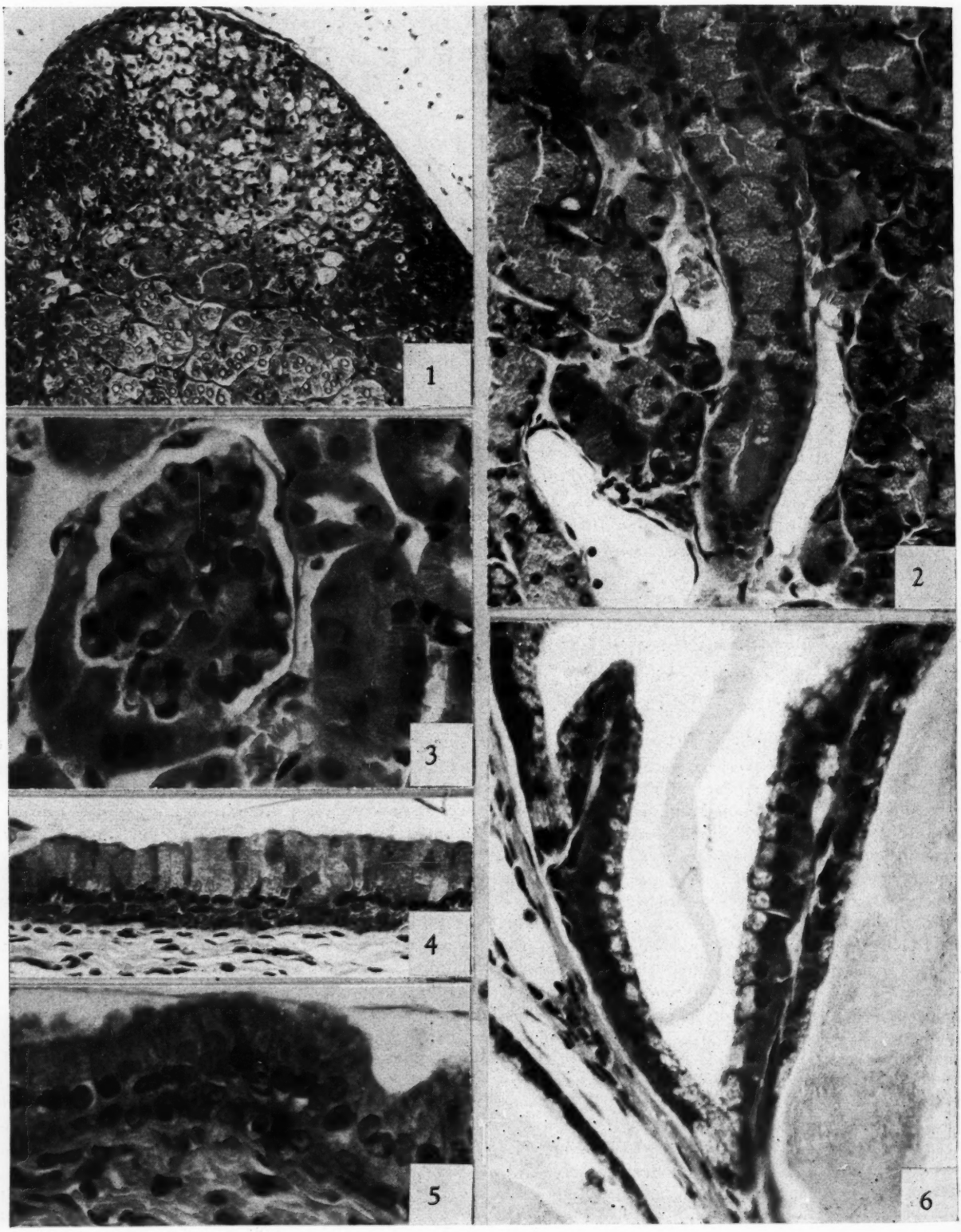
The effects of synthetic sex hormones[1] on the reproductive tract and dimorphic glands of the castrate female mouse were determined. Weanling hybrid females were ovariectomized and placed on experiment 10 days following operation when the vaginal smear was of the castrate type. Five animals comprised each of the following experimental groups:

I. 0.3 γ estradiol dipropionate
II. 70 γ testosterone propionate
III. 150 γ progesterone
IV. 0.1 γ estradiol dipropionate + 150 γ progesterone

* This investigation has been aided by grants from The National Cancer Institute, The Jane Coffin Childs Memorial Fund for Medical Research, and the Cancer Fund of the Graduate School of the University of Minnesota.

† Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Minnesota.

[1] The synthetic sex hormones were supplied by Ciba Pharmaceutical Products through the courtesy of Dr. F. E. Houghton.



FIGS. 1–6

V. 0.3 γ estradiol dipropionate + 70 γ testosterone propionate
VI. 0.3 γ estradiol dipropionate + 35 γ testosterone propionate
VII. 0.1 γ estradiol dipropionate + 35 γ testosterone propionate
VIII. No hormonal treatment. Injected with peanut oil, 0.1 cc. daily

All hormones were dissolved in peanut oil and injected subcutaneously. The dose of progesterone was in 0.1 cc., and the other hormones in 0.05 cc. peanut oil. The hormones were administered over a 21-day period, the estrogen being injected 3 times per week, the others daily. The group given progesterone-estrogen treatment was "primed" with 3 doses of estrogen. The animals were autopsied 24 hours following the last injection.

The submaxillary glands of 5 adult intact Bagg albino males were biopsied for histologic study at which time the animals were castrated. Four weeks later the mice were autopsied and the remaining submaxillary tissue observed microscopically.

OBSERVATIONS

All 10 of the castrates (Fig. 13), and 4 of the 8 intact Bagg albino males (Figs. 7, 11) 2 years or more of age exhibited tumors of the adrenal cortex. In the others subcapsular hyperplasia (Fig. 8) was present. The tumorous adrenal glands ranged in size from 2 to 3 times that of the normal gland to neoplasms ½ cm. in diameter. One of the tumors in a castrate and one in an intact mouse are to be considered carcinomas (Fig. 7). The other growths were adenomas (Figs. 11, 13). Adenomas were present in males 6 months after castration.

The adenomas were structurally similar to those described previously (4, 7, 16). They contained the fibroblast-like subcapsular "type A" cells (Fig. 8) and the lipid-laden "type B" cells (Fig. 9) of Woolley and Little (16). In the adrenals containing these tumors extensive areas of subcapsular "type A" cell proliferation were present (Fig. 8). Another characteristic of these glands was the widespread distribution of "brown cells" containing "ceroid pigment." These were found both within the adenomas and in the areas of nontumorous cortex (Fig. 12). The greatest degree of "brown cell degeneration" was in the region adjacent to the medulla.

That certain adenomas of the Bagg albino males were actively secreting androgen is demonstrated by the histology of the seminal vesicles of these mice (Fig. 14). In at least 5 of the castrate males with cortical tumors (castration 2 years previously) the seminal vesicles were of the type illustrated in Figure 14. The epithelium was tall and the lumina of the glands were filled with eosinophilic secretion. Grossly the glands were large, and on transecting them a copious secretion exuded. In 4 of these castrate males, development of the seminal vesicles was only slightly beyond the castrate condition. The seminal vesicles of the male with the cortical carcinoma were atrophic. In the castrates possessing other evidence of androgenic secretion (seminal vesicle histology), the submaxillary glands and many of the renal corpuscles were of the male type. Although cortical adenomas were present 6 months after castration in the 4 Bagg albino males examined, histologic evidence of androgenic secretion was not apparent.

Active spermatogenesis was present in the testes of the intact Bagg albino males bearing cortical tumors (Fig. 10), although sperm were not found in the epididymis and some seminiferous tubules were atrophic. Ceroid pigment was present within many of the interstitial cells and some had apparently coalesced to form giant cells. Such cytologic changes are suggestive of degenerative alteration within these cells. However, ceroid pigment was found in a large number of the interstitial cells of Bagg albino males only one year of age.

Of the 9 ovariectomized Bagg albino females

FIG. 1.—Cortical adenoma from ovariectomized Bagg albino female mouse 10 months after operation. Lipid-containing "type B" cells of Woolley and Little are present. Fibroblast-like "type A" cells are at the periphery of the adenoma. Adjacent to the medulla are giant cells containing ceroid pigment. Mag. ×70.

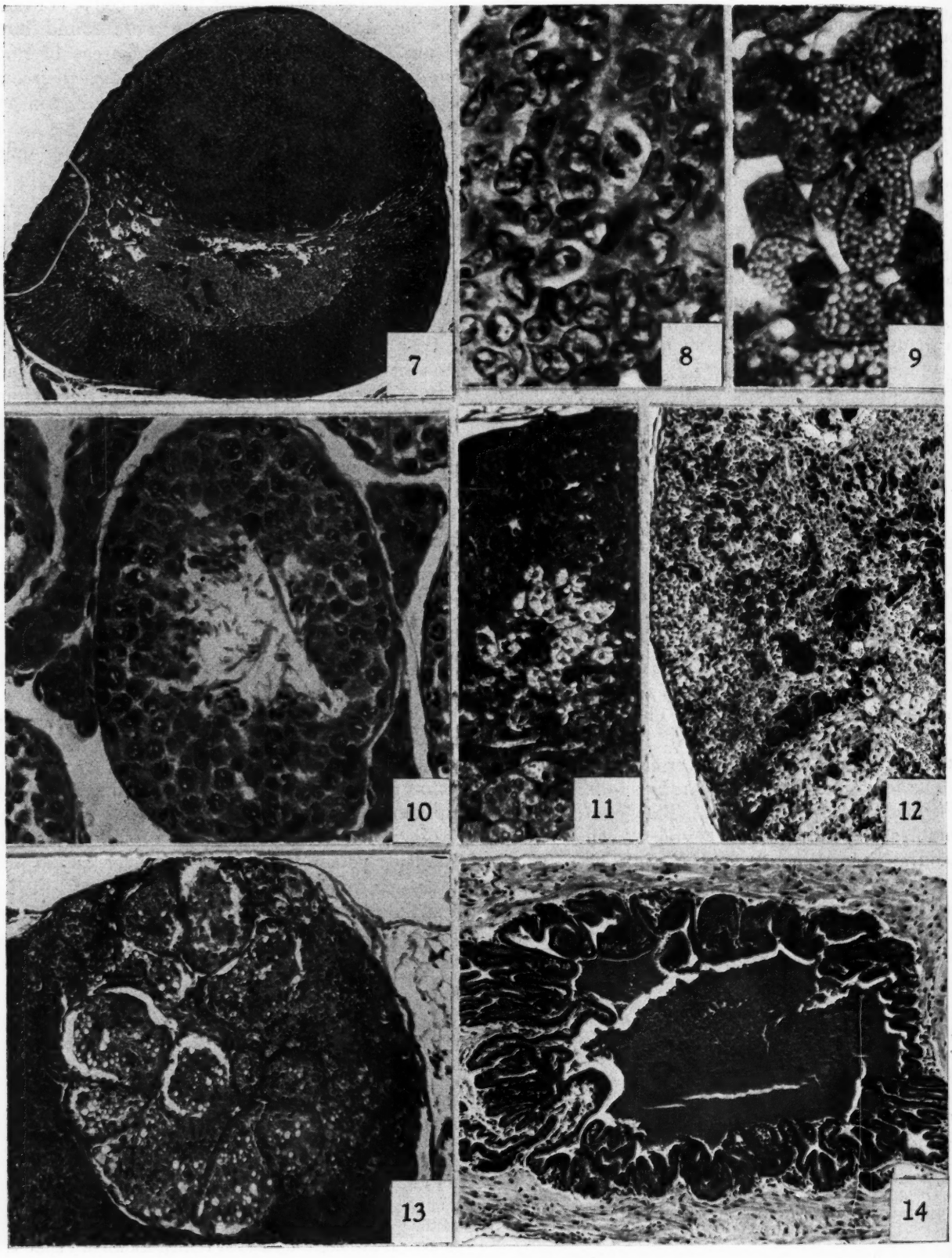
FIG. 2.—Masculinized submaxillary gland of Bagg albino female bearing the cortical adenoma shown in Figure 1. The granular cells of the terminal tubules and many of the alveoli are indicative of androgenic stimulation. In these cells the nuclei are basal. The cytoplasm is swollen and filled with pale eosinophilic secretion granules. Mag. ×250.

FIG. 3.—Renal corpuscle from kidney of same ovariectomized Bagg albino female. Cuboidal epithelial cells are present in the parietal layer. Many of the renal corpuscles were of this type indicating androgenic secretion of the cortical adenomas of this mouse. Mag. ×350.

FIG. 4.—Vaginal mucosa of same animal. The superficial epithelial cells are columnar and filled with mucoid secretion; only 2 layers of basal epithelial cells are present. Mag. ×150.

FIG. 5.—Vaginal mucosa of an ovariectomized female mouse 31 days after ovariectomy. Mouse injected with 0.1 γ estradiol dipropionate 3 times weekly for 3 weeks and 35 γ testosterone propionate daily for the same period of time. Injections begun 10 days after ovariectomy. The mucosa is histologically similar to that shown in Figure 4. Mag. ×350.

FIG. 6.—Seminal vesicle graft recovered from ovariectomized Bagg albino female with corticoadrenal tumors. The donor was a 16-day old male of the same stock. Graft made 65 days before autopsy. Mag. ×300.



FIGS. 7–14

autopsied 10 or more months after operation, only one possessed adenomas (Fig. 15) associated with an hypertrophied uterus and cornified vagina (Fig. 21). The vaginal smear was characteristic of a mouse bearing estrogen-secreting cortical tumors, that is, the smear contained epithelial cells, some of which were cornified, and leukocytes. The submaxillary gland (Fig. 16) and renal corpuscles were of the female type. The remaining 8 ovariectomized females had cortical tumors, but none possessed either large uteri or vaginae showing evidence of estrogenic stimulation. The vaginal epithelium was only 2 to 3 cell layers thick and was mucified, the superficial cells being tall columnar (Fig. 4), many of the renal corpuscles were of the male type (Fig. 3), the parietal layer of Bowman's capsule having a tall, broad epithelium. The submaxillary glands were also male (Fig. 2), the cells of the terminal tubules being filled with secretion granules, as a result of which the nuclei were flattened at the base of the cells (1, 10).

The seminal vesicle grafts which had been made into 2 of these females 2 months previous to autopsy were hypertrophied and cystic, containing a large amount of secretion. Cytologically the evidence for androgenic stimulation was conclusive (Fig. 6).

In 4 Bagg albino females autopsied 5 months after ovariectomy only subcapsular "type A" cell proliferation was present in the adrenals of 3 while the fourth had a small adenoma containing both "type A" and "type B" cells. No evidence of sex hormone secretion was apparent.

The submaxillary glands and many of the renal corpuscles were of the male type 12 months after operation in the ovariectomized CBA and C3H females possessing adenomas of the adrenal cortex. The uteri provided evidence of estrogenic stimulation, however, and the vaginal epithelium was cornified (Fig. 21). Figure 20 illustrates the masculinized submaxillary gland of an ovariectomized C3H female which exhibited vaginal cornification. A grafted seminal vesicle presented the same picture as that illustrated in Figure 6. Although seminal vesicle grafts were maintained in tumor-bearing CBA and C3H females 10 to 12 months after ovariectomy, similar grafts were not maintained in the same animals 8 months after the ovaries had been removed. At this time the adenomas were apparently secreting primarily estrogenic hormone.

In all of the castrate NH males cortical adenomas were present, but in none were the seminal vesicles enlarged. Neither the kidneys nor the submaxillary glands were of the male type. All the ovariectomized females had adenomas, in association with which there was evidence of estrogenic stimulation of the female reproductive tract. Renal corpuscles and submaxillary glands were of the female type.

Three ovariectomized Strong A females were autopsied 14 to 16 months after operation. Small adenomas were present in all. The female reproductive tract was atrophic but the submaxillary glands were masculinized. In one animal the renal corpuscles were male in type. Seminal vesicle grafts were not maintained.

Histologically the adenomas associated with androgenic (Figs. 1, 13), or mixed male and female hormonal, or estrogenic stimulation (Fig. 15) of end organs were similar.

One month following gonadectomy the submaxillary glands of male and female mice were histologically identical, both exhibiting the characteristic features of the female type (Fig. 16) as described by Lacassagne (10) and Fekete (1). Administration of synthetic estrogenic hormone to the castrate female did not alter this appearance.

FIG. 7.—Spontaneous adrenocortical tumor from intact Bagg albino male 2 years of age. This tumor contained undifferentiated cells of the type shown in Figure 8. The testis of this animal is shown in Figure 10. Mag. ×40.

FIG. 8.—Area from adrenal cortex of Bagg albino male 2 years of age. Extensive subcapsular cortical hyperplasia of this type was observed in intact aged mice of both sexes of the Bagg albino stock. These indifferent cells may form "type B" cells of the adenomas or they may become malignant. Mag. ×400.

FIG. 9.—Lipid containing "type B" cells of castration-induced cortical adenoma. Animal was perfused with Regaud's fixative and the adrenal sections were stained according to the method of Fain-Wolfe. Mag. ×500.

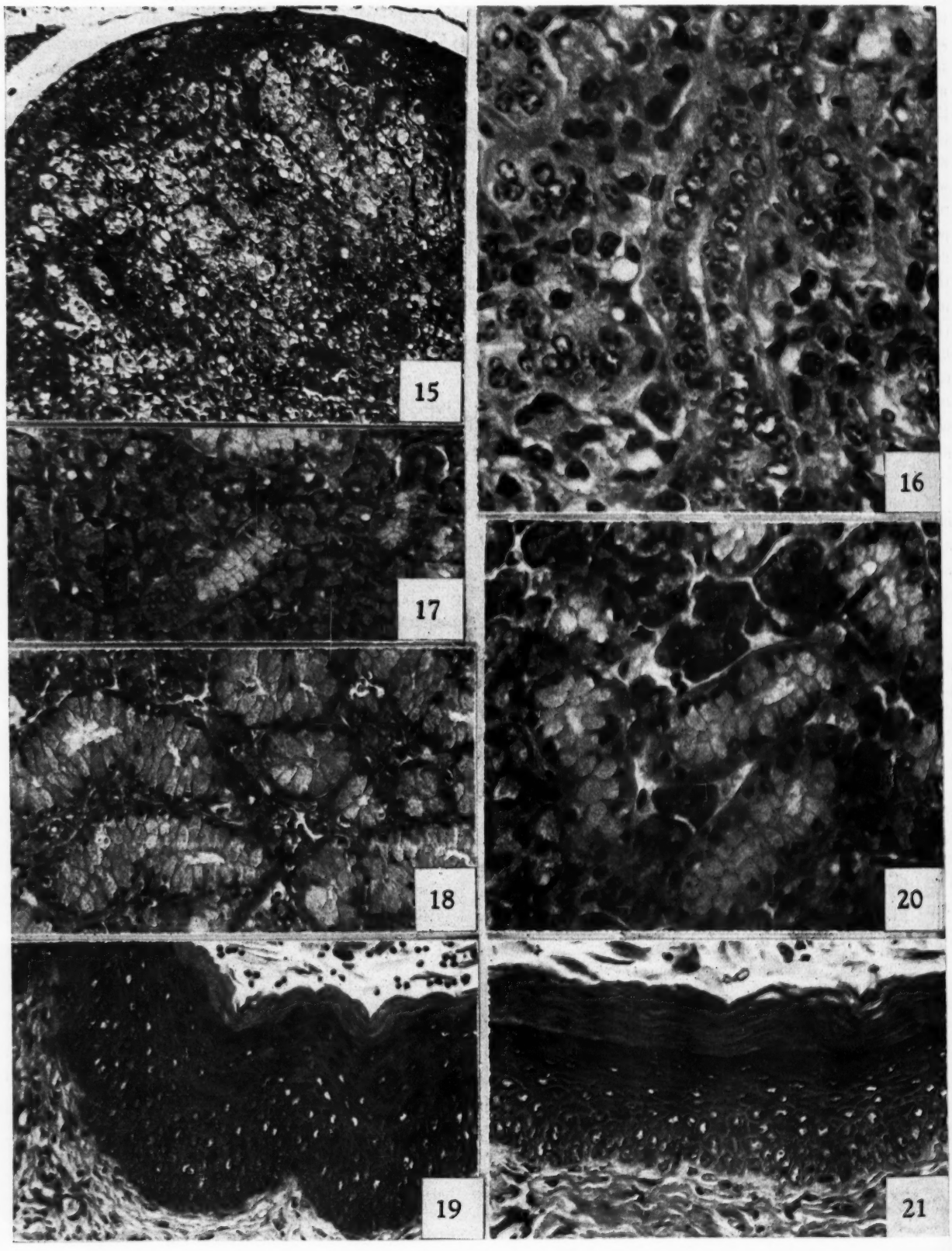
FIG. 10.—Testis of Bagg albino male bearing the cortical tumor shown in Figure 7. Females of this stock with cortical tumors may possess ovaries with occasional mature and developing normal follicles. Apparently complete withdrawal of gonadal secretion is not necessary for the development of cortical adenomas. Mag. ×300.

FIG. 11.—Adenomatous adrenocortical hyperplasia in intact Bagg albino male 2 years of age. "Type A" and "type B" cells are present. Mag. ×160.

FIG. 12.—Adrenal cortex with adenoma. "Brown-cell degeneration" present within both adenomatous and non-hyperplastic areas of the cortex. The pigment of the "brown cells" photographs black since it is stained red by basic fuchsin. Mag. ×70.

FIG. 13.—Cortical adenoma from Bagg albino male castrated 2 years before autopsy. Mag. ×70.

FIG. 14.—Seminal vesicle from castrated Bagg albino male bearing the cortical adenoma shown in Figure 13. This gland shows evidence of androgenic stimulation. Mag. ×150.



FIGS. 15–21

Progesterone, given either alone, or in combination with estrogen, produced either no change or only a slight alteration in the histology of the terminal tubules (Fig. 17). On the other hand, when androgenic hormone was given either alone, or in combination with estrogen, a masculinizing effect on the terminal ducts and alveoli was apparent (Fig. 18), the cells being filled with secretory granules, and the nuclei basal and pyknotic. The "rodded" appearance of the non-masculinized terminal ductal epithelium was lost.

The renal corpuscles of the hormone-treated mice did not reflect the androgenic stimulus to as great a degree as the submaxillary gland. Conclusive alterations in the parietal layer of Bowman's capsule were present primarily in the animals receiving the 70 γ dose of testosterone propionate.

The uteri of all animals receiving synthetic hormones, with the exception of those treated with progesterone alone, showed varying degrees of recovery from the castrate condition. Recovery with testosterone was good, whereas the uteri of those animals treated only with progesterone remained castrate. Decided stimulation resulted from treatment with estrogen, alone and in combination with the other hormones.

Vaginal mucification with thickening of the epithelium was present as a result of combined treatment with estrogen and either progesterone or testosterone, the higher dose of testosterone inducing a greater degree of mucification and "mucous-cyst" formation than the lower dose of the same hormone. With progesterone, in combination with estrogen, the typical progestational vagina of the pregnant mouse resulted. With estrogen alone typical cornification appeared. The dose of testosterone when used alone induced the development of a vaginal epithelium 3 to 4 cell layers thick, the superficial layer being low columnar and mucified.

DISCUSSION

These results demonstrate that tumors of the adrenal cortex of Bagg albino mice may secrete estrogenic and/or androgenic substance. It is likely that both types of hormone are secreted simultaneously. The mucification of the vaginal mucosa in ovariectomized Bagg albino females bearing cortical adenomas (Fig. 4) was typical of the picture induced in the group of mice receiving combined treatment with 0.1 γ estradiol dipropionate and 35 γ testosterone propionate (compare Figs. 4 and 5, see Materials and Methods). Korenchevsky (8) has produced the same picture in rats by combined treatment with estrogen and progesterone. That it is the combined action of estrogen and androgen, or androgen alone, and not progesterone, is indicated by the male type of kidney and submaxillary gland in these females (Figs. 2 and 3). Progesterone did not induce androgenic effects in the dimorphic glands of the castrate female (Fig. 17). Since the submaxillary gland was masculinized by testosterone propionate even when relatively large amounts of estrogen were administered simultaneously, it is difficult to be certain at this time whether androgen alone was being secreted by the cortical tumors of Bagg albino females. However, in the castrate females of the CBA and C3H strains bearing cortical tumors (10 to 12 months after ovariectomy) there was definite evidence of secretion of both types of sex hormone. In these animals the salivary glands were either completely male (Fig. 20) or of a mixed male-female type, as described by Woolley and Little (16), whereas the vaginae were cornified (Fig. 21). Although initially (8 months after ovariectomy) the cortical tumors of the CBA females secreted insufficient androgen to develop grafted seminal vesicles, 2 to 4 months later seminal vesicle grafts developed to the degree illustrated in Figure 6. In the Strong A ovariectomized

FIG. 15.—Cortical adenoma from ovariectomized Bagg albino female 10 months after ovariectomy. Mag $\times$70.

FIG. 16.—Submaxillary from same ovariectomized Bagg albino female. This gland is of the female type. Vaginal mucosa was similar to that illustrated in Figure 21. In this female the secretion of the cortical adenoma was predominantly estrogenic, whereas in other Bagg albino females androgenic activity predominated. Mag. $\times$350.

FIG. 17.—Submaxillary gland from ovariectomized female injected with 150 γ progesterone daily for 21 days; injections begun 10 days after operation. The gland is female in type. The maximum degree of granulation of terminal tubular epithelium contained in this gland is illustrated. Alveoli and tubules were predominantly of the female type. Progesterone in the doses used did not induce androgenic changes in the submaxillary gland. Mag. $\times$150.

FIG. 18.—Submaxillary gland from ovariectomized female which received 0.3 γ estradiol dipropionate 3 times weekly and 70 γ testosterone propionate daily for 3 weeks. The gland is masculinized. Mag. $\times$200.

FIG. 19.—Vaginal mucosa of ovariectomized female whose submaxillary gland is shown in Figure 18. Cornified epithelium. Mag. $\times$150.

FIG. 20.—Masculinized submaxillary gland of ovariectomized CBA female with cortical adenoma. Mag. $\times$250.

FIG. 21.—Cornified vaginal epithelium of CBA female whose masculinized submaxillary gland is shown in Figure 20. The vagina shows evidence of estrogenic stimulation, whereas the submaxillary gland is masculinized, indicating that the cortical tumor was secreting both estrogenic and androgenic hormone. Mag. $\times$150.

females the cortical adenomas were small. The reproductive tracts of these animals were atrophic. Although seminal vesicle grafts were not maintained, the submaxillary glands (and in one case the renal corpuscles) provided evidence of androgenic secretion. Thus, it would seem that the cortical tumors of Strong A females secreted androgenic hormone, but at a low level. In the NH stock of mice the level of androgenic secretion by cortical adenomas is not sufficiently high, if indeed this type of hormone is secreted at all, to result in growth of the seminal vesicles or masculinization of the salivary glands. In all ovariectomized females of this stock estrogenic secretion by cortical adenomas was always high as evidenced by stimulation of the reproductive tract.

Thus, the secretion of cortical adenomas was characteristic for the strain in which the tumors occurred. In the Bagg albino, androgenic secretion dominated with some indication of estrogenic activity. Cortical adenomas of the C3H and CBA strains gave evidence of mixed secretion. Those of the Strong A appeared to secrete androgen at a low level and tumors of the NH strain probably secrete only estrogenic hormone.

Inbred stocks of mice demonstrate strain differences in the development of cortical adenomas. In certain stocks, for example, C57 (14), cortical tumors are said to appear neither spontaneously nor following gonadectomy. The dba, CE, C3H (16) and Strong A stocks develop tumors after the gonads have been removed, but not spontaneously. It had been thought by the authors that the CBA and Bagg albino stocks belonged in this category until recently when tumors were observed in very old intact animals. The Strong A strain has been reported to be incapable of developing estrogen-secreting cortical adenomas (14). In the three ovariectomized Strong A females studied by the authors, androgen-secreting adenomas were observed, the salivary glands being of the male type. Finally, in the NH stock cortical tumors develop spontaneously relatively early, especially in females, as well as following gonadectomy. The onset of the tumors in this stock, at least in females, is merely accelerated by removing the gonads.

The occurrence of spontaneous cortical adenomas in intact males of the Bagg albino stock is of special interest. These tumors appear in animals with testes undergoing active spermatogenesis. Whether these animals are physiologically castrate before the adenoma develops has not been determined. Some aged males of this stock have atrophic seminal vesicles, and in one intact male with cortical adenomas no androgenic stimulation of the seminal vesicles was observed. Such observations suggest that the adenomas appear following cessation of, or diminution in, the androgenic secretion of the testis. The ceroid pigment in the interstitial cells of the testis is probably indicative of degenerative alteration. Such pigment is present, however, in the interstitial cells of breeding males one year of age. The active condition of the germinal epithelium in these intact males with adenomas might conceivably be related to the androgenic secretion of the adrenal tumors. Under certain circumstances, for example in hypophysectomized rats (11), androgen may maintain spermatogenesis. Or the active germinal epithelium may indicate that the stimulus to cortical adenoma formation, if it is gonadotrophic, is follicle stimulating hormone rather than luteinizing hormone.

In females of the NH stock the development of cortical adenomas is associated with early cessation of ovarian activity (7). Also, in mice of the Bagg albino and C3H stocks radiated with leukemogenic doses of x-rays, tumors of the adrenal cortex appeared precociously primarily in females, and only occasionally in males (6). For the females these doses of x-rays were castrating, whereas the male was not permanently sterilized nor was there any lengthy depression of androgenic secretion. Recently the authors have observed the early appearance of cortical adenomas in female Bagg albino mice in which the ovaries alone were radiated with 200 r by contact radiation. These observations, together with induction of cortical tumors by gonadectomy, strongly indicate that withdrawal of gonadal endocrine secretion is a primary factor in precipitating the development of adrenal cortical tumors in mice.

Other observations suggest, however, that complete cessation of gonadal endocrine activity is not essential for the development of adrenal cortical tumors. Subcapsular hyperplasia (Fig. 8) is apparent in the adrenal cortex of males whose testes are very likely secreting the androgen responsible for the development of their seminal vesicles. A few developing ovarian follicles may be present in Bagg albino females whose adrenals contain extensive "type A" and a smaller amount of "type B" cell development.

Females of the NH stock have cortical adenomas at the age of 12 months (3). Their estrous cycles lengthen at the age of 9 months, and although from the 10th to the 12th months adenomas are developing, at no time is the vaginal smear of the real castrate type (6). If weanling females of the NH stock are ovariectomized, it takes 3 or more months for castration-induced cortical adenomas to appear. The interval between lengthening of the NH cycle (from 9 to 10 months

of age) and the occurrence of spontaneous cortical adenomas is probably less than 3 months.

A direct effort was made to attack this problem of the relation of spontaneous cessation of androgenic testicular secretion to adenoma formation. Intact males with cortical tumors were adrenalectomized (leaving the testes) to determine whether the seminal vesicles would become atrophic. Such animals were being maintained on aqueous cortical extract. Unfortunately these mice died before definite results could be obtained.

It is the impression of the authors that ceroid pigment (brown-cell degeneration) appears within certain steroid secreting cells (adrenal cortex, ovary, testis). This is, of course, not the sole location of ceroid (12). The development of ceroid pigment within steroid hormone secreting cells can probably be interpreted as being indicative of degenerative alteration. During the course of administration of estrogenic hormone there are profound alterations in the adrenal cortex, with the cords of fasciculata cells becoming predominantly "brown cells." In the ovary, testis, and adrenal cortex of the mouse the appearance of "brown cells" is to be considered an aging process.

"Brown cell degeneration" can be associated with the development of certain endocrine gland tumors of the mouse, for example, testis under the influence of estrogenic hormone (5), ovary following radiation (6), the adrenal cortex following gonadectomy, and spontaneously (Fig. 12). Tissue death and/or injury is a preliminary phase in the development of experimental neoplasms in other locations, for example, the skin (methylcholanthrene carcinogenesis), the lymph nodes and spleen (x-ray induced lymphoma), and the liver (carbon tetrachloride-induced hepatoma).

Finally, the spontaneous appearance, or the ready induction of various endocrine tumors in the Bagg albino stock is worthy of mention. Cortical adenomas occur spontaneously in both sexes (estrogen-secreting tumors have been seen by the authors in old females), and they can be induced by gonadectomy. Ovarian and adrenal cortical tumors appear in radiated mice. And males are very susceptible to the induction of testicular tumors by the administration of estrogenic hormone (13).

SUMMARY

1. Tumors of the adrenal cortex of castrate male Bagg albino mice secreted androgenic hormone, as evidenced by the histology of the seminal vesicles, submaxillary glands, and renal corpuscles. Similar spontaneous tumors were present in non-castrate males of this stock 2 years of age.

2. The tumors induced by ovariectomy in Bagg albino female mice produced as the dominant secretion either estrogen or androgen as judged by the microscopic appearance of the female reproductive tract, submaxillary glands, transplanted seminal vesicles, and renal corpuscles. Extensive cortical hyperplasia and/or adenomas appeared in intact females 2 years of age.

3. Secretion of relatively large amounts of androgen by cortical tumors may be a strain-limited character. All of 57 castrated males of the NH stock possessed atrophic seminal vesicles, although all had adrenal tumors. Ovariectomized NH females gave evidence of only estrogenic secretion. In the CBA and C3H stocks cortical tumors of ovariectomized females exhibited mixed secretory activity, resulting in masculinization of the dimorphic glands and maintenance of grafted seminal vesicles, but cornification of the vagina. Ovariectomized Strong A female mice bearing cortical adenomas had masculinized submaxillary glands and atrophic reproductive tracts.

4. Although tumors of the adrenal cortex can be induced in certain stocks of mice by gonadectomy, and spontaneous cortical tumors appear at a time of declining gonadal endocrine activity, it appears that complete withdrawal of gonadal endocrine secretion is not essential for the appearance of adrenal cortical adenomas of mice.

5. The histology of the submaxillary gland of the mouse is a sensitive index of androgenic secretion. It is a better indicator than renal histology or maintenance of seminal vesicles. Progesterone does not duplicate the androgenic effect, and estrogenic secretion does not alter the structure of the submaxillary gland of the castrate mouse.

6. Ceroid pigment deposits are associated with the genesis of tumors of the adrenal cortex, testis, and ovary of mice.

7. The Bagg albino stock of mice is very susceptible to the induction of tumors of the steroid hormone secreting glands—adrenal cortex, testis, ovary.

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On a Special Condition of the Interphase Nucleus in Normal and Cancerous Cells*

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In the course of histological and cytological studies of tissues of mice of tumor susceptible strains (Green (5) and unpublished), an atypical condition of some of the interphase nuclei was noted. No description of the exact condition has been found in the literature. Interest was aroused by the fact that the nuclei were seen in considerable numbers in the cells of tumors as well as in nontumorous tissues. The characteristics of the condition and its high frequency of occurrence in some individuals were suggestive of a similarity to certain types of virus inclusions. Since some animal tumors are known to be caused by viruses, a study was made of both normal and pathological tissues to further define the properties of the atypical nuclei, to determine how widespread the condition is and to look for possible relationships to the development of tumors or to other specific functional states.

CYTOLOGICAL CHARACTERISTICS OF THE ATYPICAL NUCLEI

General characteristics.—Three features distinguish the atypical nuclei from normal ones in fixed preparations (Fig. 1). These are (1) the presence of considerable amounts of granular material, in addition to the usual chromatin masses and nucleoli; (2) a refractile quality of the nuclear sap; and (3) translucent hexagonal crystals, which under some conditions are birefringent. Typically all three characteristics are found in a single nucleus, which then has a highly condensed and refractile appearance. However, there seems to be a progressive but not necessarily simultaneous accumulation of the substances involved. Nuclei may be recognized as of this type which contain relatively few granules or whose sap is only slightly or not at all refractile. Crystals are not always present, but when present they may be either in condensed nuclei or in more open ones, and in the cytoplasm as well. Since there is no independent correlation apparent of either the acidophilic or the refractile condition, with or without the presence of crystals, with other factors, nuclei which show either characteristic in marked degree are considered to belong to this type, and for convenience will be referred to as A-R (acidophilic-refractile) nuclei.

FIG. 1.—Diagram of a cell in the liver of a mouse showing one normal and one abnormal nucleus. The abnormal nucleus contains coarse (acidophilic) and fine (refractile) granules, a hexagonal crystal (shown on edge, in the position in which birefringence appears) and refractile nuclear sap. There are two hexagonal crystals in the cytoplasm.

The nuclear condition is not an artifact. Nuclei that differ from adjacent normal ones can be distinguished with the ordinary light microscope in fresh mounts of living cells. They show refractile ground substance and sharply defined granules that appear dark at one focus, refractile at another. Hexagonal crystals can be distinguished but with polarized light they are not birefringent. With the phase contrast microscope (Fig. 2) these same nuclei show a bright background and fine dark granules. The refractive index of the crystals is such that their outlines are only faintly discernible. In

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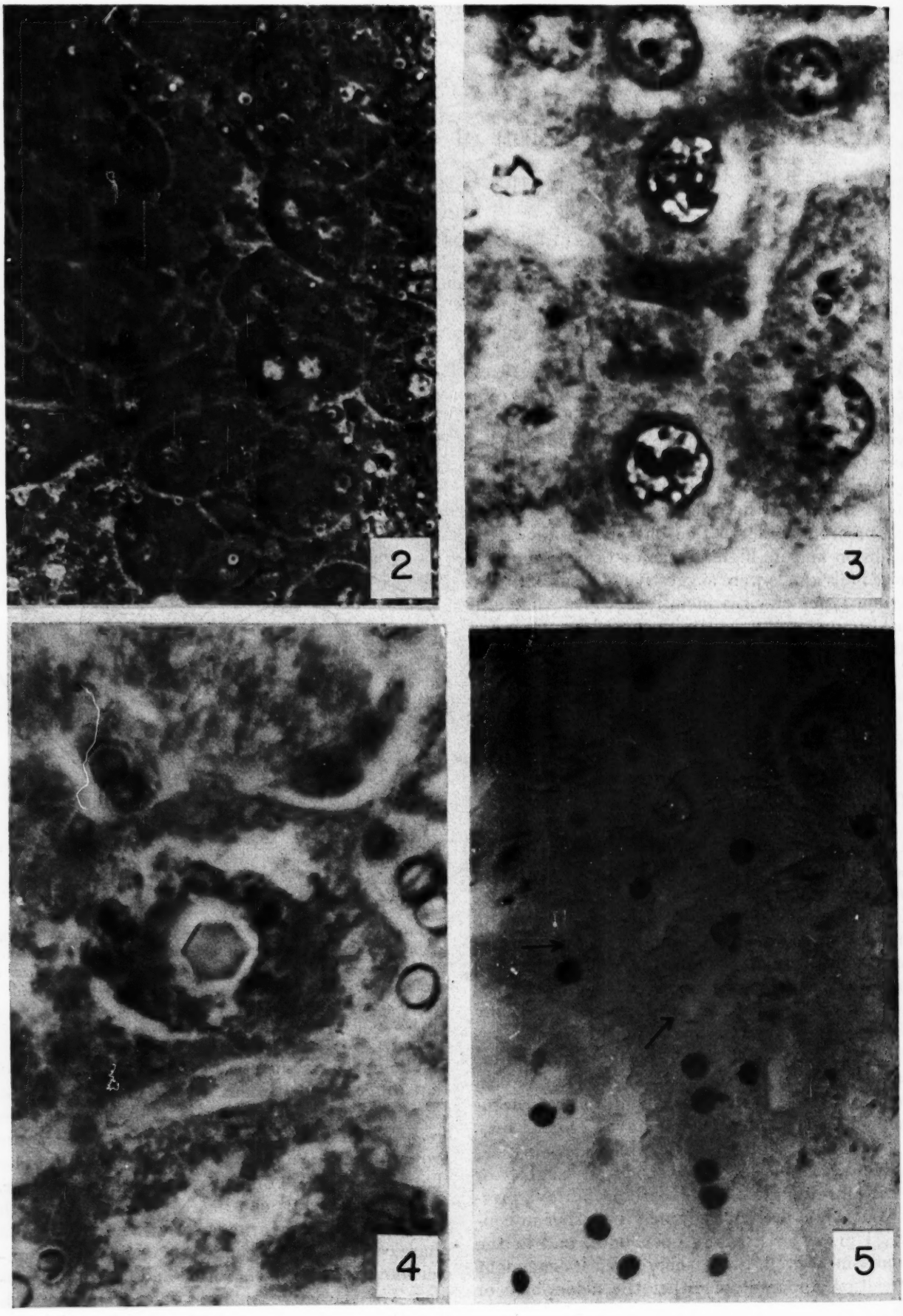

FIGS. 2–5

fixed material, the distribution of A-R nuclei is not suggestive of an artifact since it shows no relationship to the distance from the surface of the organ and therefore to the rate of penetration of the fixative or to the length of time of fixation. In all organs, cells with affected nuclei are found adjacent to those with normal ones, and in binucleate cells —for example, in the mouse liver—one nucleus may be normal, the other condensed and refractile.

The condition may be distinguished after a number of different fixatives, although some are more favorable than others for its demonstration.

Zenker's with acetic, or sublimate-acetic, or alcohol-formalin-acetic (13, 10) brings out the refractile quality of the background and the birefringence of the crystals. Zenker-formol (Helly's), Bouin's and 10 per cent formalin leave the A-R nuclei dull and the crystals rarely birefringent; however, A-R nuclei may still be identified by the staining of the granular material.

Microscopical properties of the components. Granules: The granular material comprises two components. To the first of these ("coarse granules") is due the characteristic over-all color of the refractile nuclei in stained sections. The granules take the form of irregularly shaped bodies of varying size. They are acidophilic, so that the nuclei appear pink after the Nocht-Maximow eosin-azure method or eosin-methylene blue and intensely red with acid fuchsine. With iron-alum-hematoxylin the A-R nuclei appear black almost throughout.

The second component of the granular material ("fine granules") consists of the fine particles, visible both in living cells and in unstained sections, which appear alternately dark and refractile as the focus of the microscope is changed. These particles are sometimes found in otherwise normal nuclei, often in close association with the nucleolus. In refractile nuclei they are numerous and are distributed throughout the nucleus.

Nuclear sap: The substance responsible for the refractile appearance of the A-R type nuclei is located in the nuclear sap. In unfixed cells as well as in fixed ones, both with ordinary light and with phase contrast, it may be seen to occur in varying degrees in adjacent cells or in nuclei within the same cell, where conditions are presumably similar. It does not stain with either acid or basic dyes. It is usually uniformly distributed throughout the nucleus (Figs. 2, 3).

Crystals: In their broadest cross section, the crystals have a hexagonal form with all edges in focus at the same time (Fig. 4). Occasionally other faces may be seen indicating that the form is actually a flattened rhomboid whose shortest diagonal, perpendicular to the plane of hexagonal cross section, is about one-fourth the length of either of the other two. The crystals vary in size from approximately the diameter of the nucleolus to that of the entire nucleus. They are slightly yellow in color. They are almost completely transparent and usually unstained so that they are not readily distinguishable. In some instances, the crystals stain with fast green. The large crystal shown in Figure 4 was faintly stained with eosin. Crystals appear in both refractile and non-refractile nuclei. Furthermore, they are found also in the cytoplasm. There may be several crystals in a single nucleus or in the cytoplasm of a single cell.

Examination of suitably fixed tissues with the polarizing microscope shows crystals that are birefringent. These occur only in refractile nuclei. Here the position in which the crystal lies is of importance. Although with ordinary light hexagonal forms can be distinguished, with polarized light it is only when the crystals lie at an angle to the surface of the section that birefringence appears.

CYTOCHEMICAL CHARACTERISTICS OF THE A-R NUCLEI

A summary of the cytochemical properties of the four unusual nuclear components is given in Table 1.

Coarse granules—

Protein—They do not stain with acid fuchsine after digestion of sections with pepsin (HCl pH 2.0, 4 hrs., 37° C.) or with trypsin (phosphate buffer pH 7.0 or citrate buffer pH 6.8, 4 hrs., 37° C.) but do after similar treatment with buffer alone. They continue to stain with acid fuchsine after acid hydrolysis (1 *N* HCl, 1 hr., 60° C.) but not after hydrolysis with alkali (1 *N* HCl, 45 min., room temp.).

Lipid—The material does not stain with Sudan III either before or after hydrolysis.

Nucleic acid—There is no more staining than in adjacent normal nuclei with the Feulgen reaction or with methyl green-pyronin.

Carbohydrate—There was no color in the refractile nuclei after the iodine test, the Molisch reaction, the Schiff reaction without hydrolysis, or the reaction of McManus as used by Hotchkiss (7).

Fine granules—

Protein—No fine granules were visible in nuclei digested with pepsin or trypsin or extracted with 6 *M* urea (2).

Lipid, Nucleic acid and Carbohydrate—It is not possible to say whether or not they contain lipid, nucleic acid or carbohydrate because they are so small and so

FIG. 2.—Refractile nuclei in the liver of a C57 black mouse. Fresh mount of living cells. Bausch & Lomb phase contrast microscope. Mag. ×1700.

FIG. 3.—Refractile nuclei in cells of the adrenal cortex of a C3H mouse. Zenker's with acetic. Nocht-Maximow eosin-azure. Spencer phase contrast microscope. Mag. ×2450.

FIG. 4.—Cell from the liver of a Swiss mouse showing a large hexagonal crystal in the nucleus. The nucleus contained acidophilic granules but was not refractile. Zenker's with acetic. Nocht-Maximo eosin-azure. Mag. ×1700.

FIG. 5.—Nuclei in a section of fixed (Zenker's with acetic) mouse liver showing refractile substance (dark in photograph) clumped by treatment of sections with boiling water. Normal nuclei in the same or adjacent cells can barely be distinguished (arrows). Light areas within the dark nuclei represent birefringent crystals. Unstained. Mag. ×1700.

dark or so refractile that one cannot tell whether or not they are colored by any of the reactions applied above.

Refractile substance of the nuclear sap—

Protein—The refractile material is removed by digestion of sections with pepsin or trypsin but not by buffer alone. It is also removed by extraction of tissue before fixation with 6 *M* urea (24 hrs., 5° C.) while it is present in material from the same organs fixed without extraction. Under certain conditions the substance undergoes a form of precipitation that results in the formation of a shrunken distorted mass that is brownish in color and highly refractile. This effect is seen occasionally in living cells and in fixed but unstained sections, but is enhanced by certain procedures which would be expected to affect proteins—namely, after staining by the Pappenheim-Unna method in which the dye solution contains glycerine, phenol and methanol and staining is carried out at 37° C., or in sections from which water has been evaporated by boiling to dryness (Fig. 5).

Lipid—The refractile quality is still present after treatment of sections with hot organic solvents—ethyl alcohol, ether-alcohol (1:3), chloroform-methanol (3:1), acetone, or pyridine.

Nucleic acid—The material does not stain with basic dyes and is Feulgen negative.

Carbohydrate—All of the tests mentioned above were negative.

Crystals—

Protein and Lipid—The following points indicate the presence of both a protein and a lipid: (a) birefringence is still present after digestion of sections at 37° C. with pepsin or trypsin, but disappears upon subsequent extraction with acetone (16 hrs., room temp.); indeed, crystals can then no longer be seen at all; in sections treated with buffer followed by acetone the birefringence remained; (b) no birefringent crystals are seen in sections of tissue extracted with urea as above, while the unextracted material shows considerable numbers; (c) the birefringence is destroyed by extraction of sections with the hot organic solvents mentioned above; boiling water does not affect it, nor does extraction with cold organic solvents; (d) the birefringence disappears after extraction of sections with acetone or 95 per cent alcohol at room temperature if they are first hydrolyzed with either acid (1 *N* HCl, 1 hr., 60° C.) or alkali (1 *N* NaOH, 1 hr., room temp.). On the other hand, the crystals do not stain with Sudan III either before or after hydrolysis; no positive color reactions have been obtained with Millon's reagent, the xanthoproteic, ninhydrin or diazo reactions before or after extraction with hot organic solvents; the test for arginine (13) was negative, and no color appeared with the nitroprusside test, which was tried because *l*-cystine crystallizes in a hexagonal form. While the results of these latter tests, under the present conditions, do not give positive evidence of the presence of certain chemical groupings that occur in proteins, the evidence from digestion with enzymes, from treatment with urea, from extraction with organic solvents with and without hydrolysis strongly suggests the presence of protein combined with lipid.

Nucleic acid—The crystals are not stained by toluidine blue or by methyl green-pyronin and they are Feulgen negative. The reaction of St. Hilaire for purines (8) was negative, and the crystals are not blackened by $AgNO_3$.

Carbohydrate—All of the tests used above were negative.

DISTRIBUTION AND INCIDENCE OF A-R TYPE NUCLEI

In order to evaluate the significance of the fact that nuclei of the A-R type are found in tumors, it seemed advisable first to investigate their distribution generally with respect to species of animals and to organs and tissues within the individual. The incidence in a single organ was examined more thoroughly in order to determine the variability among individuals and to look for possibilities of relationship to normal factors such as age, nutritional condition or growth and cell division. The condition was also considered in relation to certain general pathological features with which it seemed to be associated, and finally with respect to its occurrence in tumors and tumor susceptible animals.

TABLE 1

CHEMICAL CONSTITUTION OF COMPONENTS OF A-R TYPE NUCLEI

	Protein	Lipid	Nucleic acid	Polysaccharide
Coarse granules	present	probably absent	absent	absent
Fine granules	probably present			
Refractile component of nuclear sap	present	probably absent	absent	absent
Crystals	present	present	absent	absent

General distribution of A-R nuclei.—The nuclear condition has been found to occur in several vertebrate species.

Slides of human organs fixed in Zenker-formol, examined through the courtesy of Dr. Clark E. Brown, showed refractile nuclei in considerable numbers in one individual, in smaller numbers in several others. Material obtained from a 3 months human foetus and fixed in Zenker's fluid with acetic showed refractile nuclei in every organ examined—liver, kidney, adrenal, intestine, lung and spleen. They were present also in the uterus and placenta of the mother. A Syrian hamster which died showing congestion of the lungs, and 2 rabbits which were freshly killed, showed considerable numbers of refractile nuclei in several organs. Properly fixed tissues from rats and guinea pigs have not been examined. The condition was also found in both tadpoles, and adult

frogs of the species *Rana pipiens* and in a bullfrog, *Rana catesbiana*.

Nuclei of the A-R type have been seen in mice of the Swiss, C3H, A, Bagg albino, C, dba, C57 black, "62" (Lynch), and "5" (Lynch) strains. No marked strain differences in incidence have been noted. Neither is there any sex difference.

The most extensive study of the incidence has been made in the C3H and Swiss strains. Here nuclei of the A-R type have been found at some time in every organ examined. Frequently they are prevalent in both the parenchymal cells and the connective tissue but if they are absent from the former they are still found in the latter. Here they occur in all types of cells—fibroblasts, macrophages, endothelial and reticular cells and in lymphocytes. One gains the impression that the connective tissues may act as a reservoir from which the effect spreads at times into the functional cells of the organs.

Because of its homogeneity and the relatively large size of its nuclei, the liver was chosen as a representative organ in which to observe the incidence of the A-R nuclei.

Counts were made on a single section (6μ) from a piece of liver taken in each case from the left lateral lobe. The average number of refractile and/or completely acidophilic nuclei per microscopic field (obj. 44×, oc. 15×) was obtained by counting adjacent fields in rows spaced 1 mm. apart. Counts on more than one section from a single animal were in agreement, on the average, within less than 10 per cent.

The total number of nuclei, normal and abnormal, included in a single count was found to range from 1000 to 7000. Sample counts on sections of liver from mice of different ages showed that there was an increase in cell size and hence a decrease in the total number of nuclei per field with age. The decrease, exponential in form, runs from about 90 nuclei per field at 1 month to 35 at 20 months. The curve thus obtained was used in estimating the percentage of the total number of nuclei represented by actual counts of the number of A-R nuclei.

Incidence of A-R type nuclei with respect to age.—The mice from which liver counts were made range in age from foetal stages to 22 months. A-R nuclei are found at all ages. Individual counts from 135 stock mice of several strains are shown in Figure 6. It can be seen that there is a wider range of variability in young mice than in older ones. The proportion of counts higher than 5 A-R nuclei per microscopic field is significantly greater in mice 5 months of age and younger than in mice that are over 5 months (χ^2 test, $P < 0.001$). There is also a decline in the successive median values at each age.

At each age, the counts range from 0 upward and there are more low values than higher ones. Therefore, the median is a better representation in each instance than the mean. It is necessary also to take into account the decline in total number of nuclei per field with age mentioned above. The logarithmic lines of the medians of both A-R nuclei (B) and total nuclei (A) are shown in Figure 6. It can be seen that the rate of decline in A-R nuclei per field is faster than can be accounted for by the decrease in total nuclei per field until a level is reached after which no further falling off occurs.

The decrease in variability and the decline in median values with age shown in the composite group of stock mice can be seen in smaller groups of mice of a single strain.

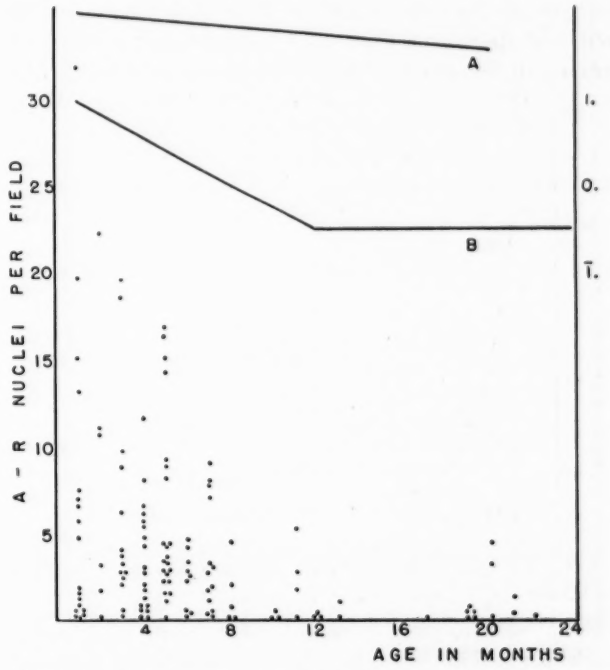

FIG. 6.—Counts of A–R type nuclei per microscopic field in the livers of freshly killed stock mice. Lines A and B represent the logarithms of the median values of total nuclei and A–R nuclei per field respectively at each age.

The relatively high incidence of A-R type nuclei in young mice and their presence in foetal stages might indicate that there is a correlation with the actively growing phase of organs and tissues. One piece of evidence suggests that this is not the case. In the mouse intestine, the frequency of A-R nuclei is lower in the deeper portions of the glands, where mitoses are common, than in the more differentiated superficial epithelium.

The variability of counts in a group of animals of uniform age, strain and environmental experience proves to be great. Since, as already indicated, there are usually more low values than high, the frequency distribution of the counts is not normal, and may be so skewed as to be exponential in form (Fig. 7A). When there are a larger proportion of high counts, it may take the form shown in Figure 7B. Therefore, in comparing the incidence in

different groups, those statistical tests which depend upon a normal distribution are not applicable.

Incidence of A-R nuclei with respect to nutritional condition.—If the nuclear condition is related to some phase of the normal metabolism of the cell it must be to a general and not a specific process, since it is found in the parenchymal cells of many organs with different specific functions and in cells of the connective tissue as well.

In order to see whether the incidence of the atypical nuclei is directly related to the state of nutrition of the cell, a group of C3H mice were left without food for 24 hours. These animals lost 2 to 4 grams in weight and at autopsy showed fatty livers. Nevertheless, the counts of A-R type nuclei in the liver did not differ in range or distribution from those of litter mate controls which had a continuous supply of food.

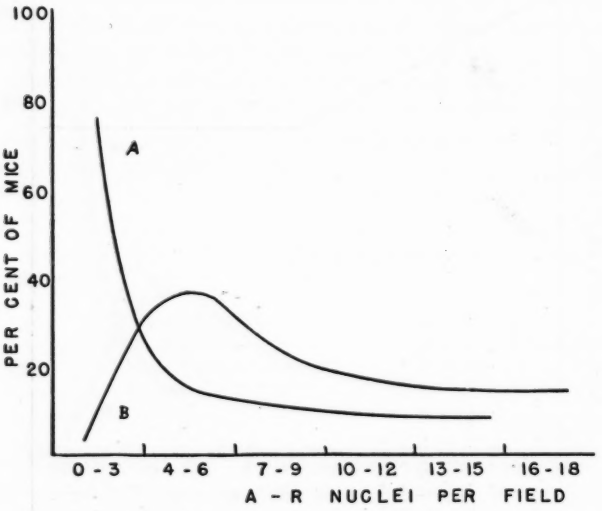

FIG. 7.—Frequency distributions of counts of refractile nuclei per field in the livers of mice of the same age. Curve A represents the type of distribution found in six groups of mice; curve B shows the type of distribution in five other groups.

In the livers of mice generally there is no apparent relationship of the position of cells containing A-R nuclei to blood vessels. The nuclei in a binucleate cell in the liver would presumably be in a similar condition in so far as their dependence upon substances entering by way of the cytoplasm is concerned, yet frequently one and not the other nucleus is affected. Therefore there is no evidence to suggest that the presence of the A-R type of nucleus is determined by the nutritional state of the cell.

RELATIONSHIP OF A-R TYPE NUCLEI TO PATHOLOGICAL CONDITIONS

In the foregoing sections, the incidence of A-R type nuclei has been considered with respect to various factors that are normal or physiological in nature. The possibility also exists that they may be related to pathological conditions.

Association with general pathological features.—While the widespread occurrence of the A-R type nuclei in young and apparently healthy animals and in cells whose cytoplasm seems unaffected indicate that the nuclear condition, in certain of its phases, is not detrimental or degenerative in nature, nevertheless, one gains the impression from histological observations that there is a consistent set of pathological features with which the presence of large numbers of A-R nuclei is associated.

In organs of apparently healthy animals, in which there are considerable numbers of A-R nuclei, either general or localized areas are found which show these features. Here the cytoplasm of the parenchymal cells is more acidophilic than in neighboring regions; there is congestion of capillaries and small blood vessels, or even diffuse hemorrhage; frequently there is diffuse infiltration with lymphocytes, many of whose nuclei are in the refractile or acidophilic condition; polymorphonuclear leucocytes are absent; the cytoplasm of macrophages in the affected organ often contains a pigment-like substance that is greenish gray in eosin-azure preparations. In the brains of apparently healthy animals, refractile nuclei are seen occasionally in the glia or ependymal cells. Groups of nerve cells in the cerebral cortex or of Purkinje cells in the cerebellum frequently show shrunken basophilic cytoplasm and nuclei that are deeply acidophilic throughout, although not refractile. Translucent hexagonal crystals are present in both the nuclei and the cytoplasm of these cells. Neighboring nerve cells are normal in appearance or may contain diffusely scattered acidophilic granules. While congestion has been noted only rarely in these brains, there is sometimes diffuse infiltration with lymphocytes, particularly in the granular layer of the cerebellum.

The foregoing features are especially prominent in certain animals that have died showing relatively high counts of A-R nuclei in the liver. Gross symptoms have been diarrhea and/or a nodular type of pneumonia. No bacteria are found, either histologically or in cultures except in the intestine. In the organs of these animals, the A-R nuclei seem to have undergone pycnosis. They stain more heavily with basic dyes—they contain much Feulgen positive material—yet still show the refractile quality of the background, the acidophilic and refractile granules and birefringent crystals. (A check was made to see that the high numbers of A-R type nuclei in the mice that died was not due to postmortem changes. A mouse was killed and allowed to remain at room temperature. Sections from pieces of liver fixed after 2, 4, 6 and 24 hours showed no increase in the number of A-R nuclei).

Another type of degenerative transformation is seen in certain nuclei that contain hexagonal crystals but are not refractile (Fig. 8). While a few masses of chromatin are applied against the nuclear membrane, the nucleoli and remaining chromatin are clumped in the center of

the nucleus about one or more hexagonal crystals. The relatively clear zone between the central mass and the nuclear membrane contains some small eosinophilic particles and a number of refractile granules.

Two other formations have been noted which may or may not be associated with the A-R condition of the nucleus. (1) In the livers of stock mice, there may be seen in as many as 2 per cent of the nuclei discrete inclusion-like bodies. These vary in appearance from small brilliantly eosinophilic globules to large pale-staining spherical bodies or even vacuoles containing diffuse eosinophilic material (Fig. 9). Similar bodies have been described by Shibatsuji (14, 15) in C3H mice. Sometimes a vacuole or homogeneous inclusion lies outside of but adjacent to the nucleus. (2) In dividing cells, basophilic granules have been noted in the cytoplasm around and within the spindle area. The granules appear after fixatives that do not preserve the mitochondria. They are not Feulgen positive. They are conspicuous in tumor cells, but have been seen also in dividing cells in normal tissues.

If the features described above constitute a consistent pathological syndrome, the A-R nuclear condition may represent a general physiological response attributable to a number of different agencies, or it may indicate the persence in the cell of a specific infectious agent. The presence of acidophilic granular material is suggestive of a virus. Although crystals have not often been reported in virus infected animal cells, they are characteristic in one instance.

The condition described here resembles that found in the polyhedral disease of silkworms (4) in that the nuclei contain crystalline bodies accompanied by fine granules, but differs in that here neither the crystals nor the granules give a positive test for nucleic acid. In its distribution with respect to species, organs and types of cells affected, as also in the presence of acidophilic granular material and of basophilic "minute bodies," the appearance of the A-R nuclei is suggestive of herpes simplex,[1] but intranuclear and cytoplasmic crystals and a refractile substance in the nuclear sap have not been reported for herpes.

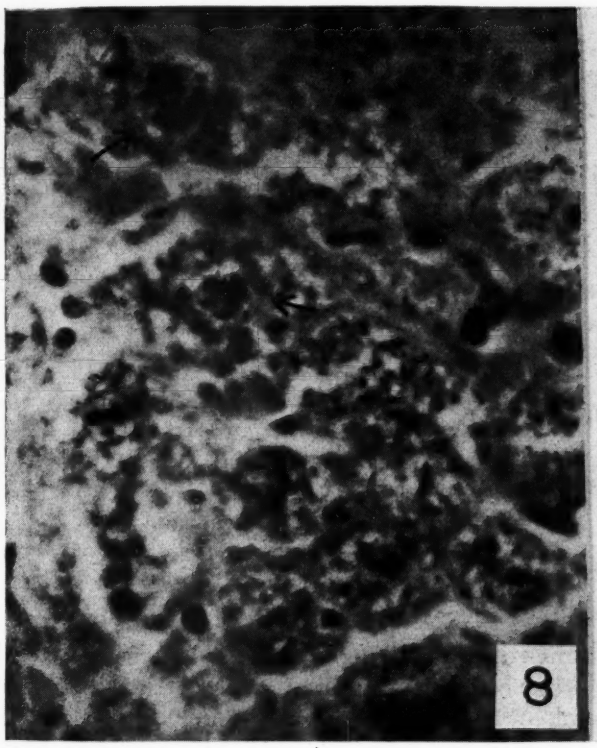

FIG. 8.—Nuclei from the liver of a Swiss mouse 21 months old showing clumping of chromatic material about a hexagonal crystal. Zenker's with acetic. Nocht-Maximo eosin-azure. Mag. ×1700.

FIG. 9.—Nucleus from the liver of a Swiss mouse 21 months old showing five inclusions. Zenker's with acetic. Nocht-Maximow eosin azure. Mag. ×1700.

[1] Homogeneous inclusion bodies of the type characteristic of herpes were present in the adrenal cortex in the 2 rabbits examined, together with large numbers of A-R type nuclei. This is a location where inclusions occur characteristically in rabbits infected with herpes. The 2 rabbits were in an experiment not the author's in which they had received repeated doses of bacterial polysaccharide followed by adrenal cortical hormone. It is probable that a fever had been induced, and herpes lesions are known to appear in individuals with high or prolonged fever.

The occurrence of A-R type nuclei in the frog as well as in mammals suggests that if the condition is due to a virus it must be one of low specificity, but it need not be less specific than the virus of psittacosis, which, although native to birds, is infective for man.

Incidence of A-R type nuclei with respect to tumors.—Nuclei of the A-R type are found in all types of tumors that have been examined. These include spontaneous, induced and transplanted tumors and carcinomas as well as sarcomas. They are found in fibroblasts and other cells of the stroma and also in varying percentages of the tumor cells themselves. Here, as in other locations, the nuclei may be very refractile and granular, or they may be non-refractile but acidophilic in varying degree. Hexagonal crystals are found, not only in the refractile and/or acidophilic nuclei, but also in nuclei that are vesicular in appearance and contain only small amounts of acidophilic material. The crystals may often be seen in dividing cells, lying either among the chromosomes at metaphase or anaphase or in the cytoplasm. They occur with considerable frequency also in the cytoplasm of non-dividing cells.

TABLE 2

INCIDENCE OF A-R TYPE NUCLEI IN TUMORS AND ADJACENT NORMAL TISSUE

Animals	Number	Tumor	A-R type nuclei	Normal tissue	A-R type nuclei
C3H mice	3	liver—spontaneous hepatic cell	7–33%	liver parenchyma	9–25%
	7	mammary gland—spontaneous adenocarc.	0.2–28%		
	4			mammary epithelium	7–17%
Swiss mice	3	lung—spontaneous	26–36%	alveolar cells and bronchial epithelium	22–34%
	6	lung—1, 2, 5, 6-dibenzanthracene	7–50%	alveolar cells and bronchial epithelium	10–59%
Frog	1	kidney—spontaneous adenocarc.	a few	kidney parenchyma	considerable

In cases where it was possible to make counts of A-R type nuclei in the tumor and in adjacent normal tissue, as shown in Table 2, the incidence in the two locations proved to be closely similar. The correlation between tumor and normal tissue in a single animal is close, whereas there is considerable variation in different animals with the same type of tumor. Spontaneous tumors of the mammary gland in C3H mice show greater variation among fields of the same tumor than the lung or liver tumors.

We have seen that A-R type nuclei are found in both mammary tumors and normal mammary glands of mice with the mammary tumor milk agent. In view of the fact that the agent is known to be at times widely distributed throughout the animal (1), tissues were examined from mice with and without the milk-factor of both C3H and C strains (obtained through the kindness of Dr. H. K. Andervont). A-R nuclei were found in a number of organs, including the mammary gland, of both milk-factor and non-milk-factor animals, and liver counts showed a similar incidence in the two groups. These results indicate that the occurrence of A-R type nuclei is not dependent upon the presence or absence of the milk agent.

Mice of the C3H and Swiss strains, which differ markedly in their tendency to develop spontaneous tumors in the lung, show approximately the same range of percentages (5 to 60 per cent) of A-R nuclei in the alveolar cells and the bronchial epithelium. One may say in general that although the A-R type nuclei appear in various tumor-susceptible organs—as, for example, the livers of C3H mice, the salivary glands of strain C mice, and the lungs of Swiss, Bagg and strain A mice—yet their presence in a given organ does not necessarily result in the appearance of a tumor, since they also occur in the lungs of C3H, dba and C57 black mice, the livers of Swiss mice and the salivary glands of Swiss and C3H mice, where tumors are rare. The abnormal nuclei are widespread throughout the individual, yet tumors tend to appear in only one or at most a few organs. In spite of the fact that all of the characteristics associated with the A-R nuclei are found with considerable frequency in tumor cells, the evidence from their incidence does not give a decisive answer concerning a possible relationship of the nuclear condition to the origin of tumors.

DISCUSSION

In the literature there are descriptions of the independent occurrence of certain intranuclear structures similar in some respects to those described here.

Acidolphilic material has been reported in several connections. Heidenhain (6) described acidophilic granules in nuclei of amphibian and mam-

malian cells which he called "oxychromatin." Pfuhl (11) illustrates a difference in staining of the nuclei in binucleate cells in the livers of rabbits, where one nucleus is sometimes more acidophilic than the other. Acidophilic bodies are described by Scharrer and Scharrer (12) in nerve cells of certain locations in the brain of fish, reptiles and man which they regard as secretory in nature. Cowdry (3) states that "acidophilic material normally occurs in a great many nuclei, especially in those of nerve cells." It is also characteristic—either in diffuse form or as formed inclusions—of the presence of intranuclear viruses.

Crystalline bodies have been seen occasionally in animal cell nuclei. Weatherford (16) reviews the literature. The most extensively investigated crystals are those found in the livers of dogs and other Canidae. Weatherford regards the canine crystals as probably bound up with purine metabolism, although he discusses the possibility of association with a virus (17). He also finds homogeneous inclusion bodies in the liver nuclei. The crystals in the Canidae differ from those described here in that they are elongated prisms, stain regularly with acid and basic dyes, and were not birefringent under the conditions under which they were observed. They were found only in the liver and the kidney, and never in more than 2 per cent of the nuclei. Like the crystals described here, the crystals studied by Monné (9) in the nucleus of the sea-urchin egg give evidence of being composed of lipid and protein. They differ in that they are rod-shaped rather than hexagonal; are birefringent before but not after fixation; are not preserved by fixatives containing acetic acid; and after suitable fixation, stain with iron-alum hematoxylin.

No report has been found of a refractile condition of the nuclear sap or of the formation of a refractile precipitate.

The association of the characteristics described here as constituting a composite nuclear condition seems not to have been recognized previously. The question at once arises as to its nature and what may cause it. Three possibilities present themselves: (1) either the condition may be involved in a particular pathological process, such as the development of tumors; (2) it may be the visible expression of a more general type of pathological response, or be due to a specific infective agent; or (3) it may represent an accumulation of normally occurring substances.

The occurrence of the A-R nuclei in several types of spontaneous tumors and in induced and transplantable tumors as well suggested a possible relationship between the nuclear condition and the development of tumors. Since the strains of mice primarily examined were known to carry the mammary tumor milk factor, it seemed possible that the nuclei might represent the location of this agent. However, nuclei of this type were found also in mice known not to carry the milk factor. Subsequently, the nuclei were found to occur with equal frequency in normal tissues adjacent to the tumors and in organs of animals which are not ordinarily susceptible to the production of tumors in that organ. On the one hand, it cannot be said that their presence will be followed by the appearance of a tumor. On the other hand, they are of such general occurrence that it cannot be stated at present whether or not a tumor can arise without their presence. Thus, the possibility is not ruled out that, given the proper combination of secondary factors, such as hormonal balance, genetic susceptibility, and nutritional requirements, the condition may be related to the genesis of tumors.

If the condition is not related to the production of tumors, it may represent a general type of pathological response on the part of the cell, or it may indicate the site of activity in the cell of one or more agents of extrinsic origin. The impression from histological observations of association of the nuclear condition with a pathological syndrome is a strong one. The variability in incidence of affected nuclei in mice that are relatively uniform genetically and in environmental experience is suggestive of individual differences in susceptibility to some pathological process. The higher overall incidence in young than in older mice would fit with a developing immunity. The constituents that appear in the affected nuclei are suggestive of those found in certain virus diseases. Neither the occurrence of the affected nuclei in apparently healthy animals nor their wide distribution need rule out the possibility of an infectious agent, since a number of viruses are known to occur in latent form and, in some instances, to show a very low degree of specificity.

The possibility also exists that the substances which appear to accumulate are of normal occurrence and that under certain physiological conditions as yet undetermined they are produced in greater amounts. In favor of this hypothesis speak the wide distribution of the condition with respect to species, organs, tissues and cell types; its appearance in young and apparently healthy animals and in cells that otherwise appear normal; the presence of the hexagonal crystals in dividing cells, indicating that they are not necessarily detrimental to the cell. If these are normally occurring substances, it is perhaps surprising to find such variability in their incidence. If the condition were supposed to represent a normal form of differentiation

of the nucleus and hence a special phase of nuclear activity with respect either to cell division or to cell metabolism, a predictable distribution might be expected with respect to some factor or factors such as organs, position within the organ or definite stages in a cell cycle.

On the basis of the data now available it is not possible to say into which of the three categories mentioned above the functional role of the A-R type nuclei will fall. The point of significance at present is the establishment of the characteristics of the A-R nuclei as a definite nuclear condition or syndrome.

SUMMARY

1. An unusual condition of the interphase nucleus has been observed in tissues of man, rabbit, hamster, mouse and frog.

2. In addition to the usual constituents, the nuclei contain acidophilic granules, fine refractile granules, a refractile element in the ground substance, and birefringent crystals.

3. Cytochemical tests indicate that all four of these components contain protein. The crystals probably contain lipid as well.

4. Nuclei of this type are found in all organs and in derivatives of all cell layers.

5. The incidence of affected nuclei in the livers of mice shows a decrease in variability and in median values with age. It is not influenced by 24 hour fasting.

6. The condition is considered with respect to certain pathological features that seem to be associated with higher numbers of affected nuclei and to certain virus diseases.

7. The incidence in tumors is closely similar to that in adjacent normal tissues, and the nuclei appear in organs of animals that are not ordinarily susceptible to the production of tumors in that organ as well as in those that are.

8. It is not possible to say what causes the nuclear condition, but its wide occurrence suggests a process of some functional significance.

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Development of Leiomyomas in Female Rats with an Endocrine Imbalance*

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Smooth muscle tumors of the uterus are rare in rats. In our colony such tumors have never been found. It is true, however, that only a fraction of the colony has been allowed to reach extreme old age before autopsy. Bullock and Curtis (1) have data on over 2450 rats with cysticercus tumors and 489 rats with independent or primary spontaneous tumors. In the latter group there were 48 uterine tumors of which 2 were myosarcomas and 4 were leiomyomas. Therefore, the presence of leiomyomas in 4 of 16 endocrine imbalance female rats that had survived more than 3 years seems well worth reporting.

MATERIAL AND METHODS

One hundred and eighty-five newborn female rats received testis grafts in the neck region by the technique of Pfeiffer (6). The method of administering the cold anesthesia in this technique has been gradually modified so that the animals are no longer chilled in the refrigerator but instead are placed on the ice in the freezing trays which are set out at room temperature. As soon as the animals become unresponsive to tactile stimuli, which occurs in a very few minutes, they are removed and quickly operated. If they begin to respond to pain stimuli before the operation is completed they are placed back on the ice and the operation is finished there. If this is not convenient they are allowed to cool down again and removed to finish the operation. With a little practice the animals can be placed on the ice a few at a time so that deep anesthesia is attained at almost the exact time that the animal is needed for operation. The short exposure to cold and the rapid return to normal temperature greatly reduces the mortality.

The donor was always a littermate of the host. Usually 2 testes were transplanted into each female (142 cases), but due to the variations in the sex ratio in the litters, the number of testes grafted ranged from 1 to 4. It has been shown that 1 testis graft, if it takes well, is sufficient to produce the endocrine imbalance (6) but 2 or more increase the chance of a good take. Subsequent to weaning, these animals were fed a commercial dog chow diet which varied somewhat throughout the experiment due to wartime conditions and animal food shortages. However, they were kept under the same conditions as the rats in the breeding colony which served as controls. All tissues were preserved in Bouin's fluid, imbedded in paraffin, sectioned at 8 μ and stained with Erlich's hematoxylin and triosin or mordanted with Zenker's fluid and then stained with either Mallory's or Masson's connective tissue stain.

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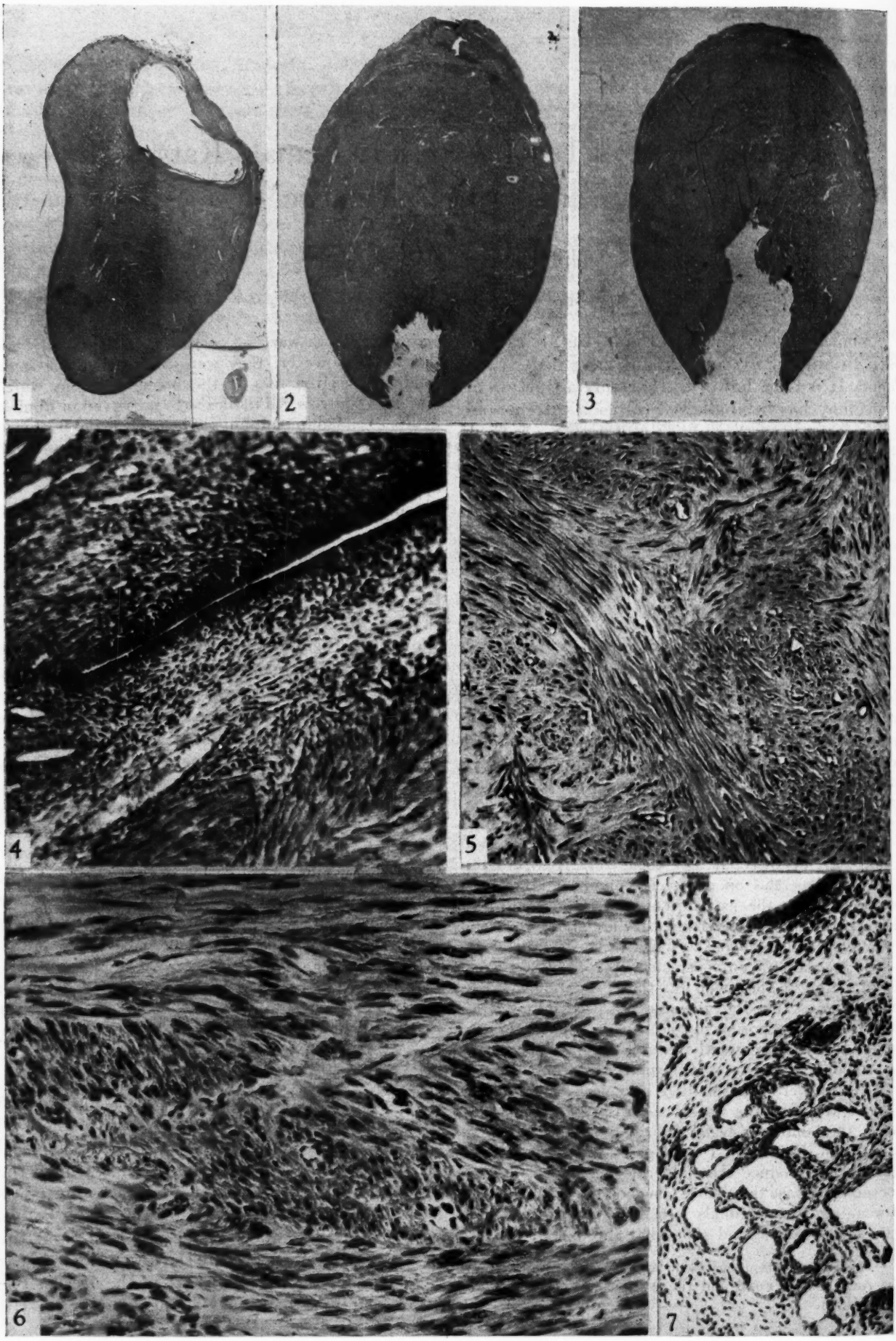
OBSERVATIONS

An endocrine imbalance is usually established in the female rats with testis transplants by the time they reach sexual maturity. This fact is expressed by the presence of constant estrus as shown by Pfeiffer (6). Vaginal smears were not followed continuously during the present study because the constant estrus condition has been well demonstrated by the hundreds of animals of this type described previously (6, 7, 8), and used in unpublished investigations. Pyometra is usually observed in these animals beginning after 6 months of age and becoming progressively more common as the period of constant estrus lengthens. The condition becomes increasingly severe until the major part, or even the whole, of the genital tract is involved. As the pyometra becomes more extreme, the uterus ulcerates into the body cavity, forms adhesions and often ulcerates through the body wall to the exterior or into the digestive tract, resulting in the death of the animal. Very few animals survive the pyometra, but there is some evidence, although proof is lacking, that all the animals that do live until senility have at some time had pyometra.

Of the 185 animals under observation only 16 survived for more than 3 years. Four of these 16 animals had uterine tumors which on histological examination proved to be leiomyomas. One of the animals had 3 tumors; one (25 $\times$ 15 mm.) was located in the lower third of the right cornu, another (10 $\times$ 8 mm.) was situated in about the middle of this horn, and the third was located near the cervical end of the left horn of the uterus. The other 3 animals each had a single tumor; in 1 it



FIGS. 1–7

was in the right horn and in the other 2 it was in the left horn. In all of these animals there was evidence of pyometra.

All of the tumors except the largest one were eccentric in respect to the rest of the uterus, a condition which indicates that the tumor started as a localized area in one side of the uterus. In the animals which did not have any tumors the uterine wall was somewhat thickened. This was especially evident in comparison with the uteri of old normal female rats. The ovaries of the animals with tumors had much the same gross appearance as the ovaries of senile animals. There was no luteal tissue that could be detected macroscopically. There was, however, some evidence that luteal function was not completely suppressed in some of the animals which had not developed tumors. Occasionally a remnant of a testis graft was found at the time of autopsy.

With hematoxylin and triosin stain all the tumors appeared to be composed entirely of smooth muscle cells. However, with connective tissue stains (Mallory's and Masson's) it was seen that varying amounts of connective tissue were present. The smaller tumors were composed entirely of smooth muscle (Fig. 6), but as the tumors became larger, fine collagenous fibers became interspersed among some of the muscle fibers. There was a gradual increase in the fibrous tissue until in the largest tumor it greatly predominated, with large areas of connective tissue separating the smooth muscle portions of the tumor. There were also large areas of mixed muscle and connective tissue (Fig. 5). The tumors had the appearance that as they increased in size the proliferation of connective tissue greatly exceeded that of smooth muscle.

On the mesometrial side of the largest tumor (Fig. 1) there was a fairly large necrotic area which was presumably the former lumen of the uterus and is interpreted as having resulted from pyrometra. The thin wall on the mesometrial side had the same appearance as the subserosa of any uterus in which there is marked pyometra. On the opposite side the necrosis had extended to the tumor tissue, leaving no indication of an intervening endometrium. The tumor was well vascularized, and there was no evidence of necrosis in the tumor proper. It had apparently grown slowly, allowing complete organization of muscle bundles and vascular channels, both of which became separated by large amounts of connective tissue. The tumor was completely encapsulated, and there was no evidence of invasion or metastasis. From the histological appearance of the tumor it is unlikely that it would ever have become malignant.

The four smaller tumors showed very clearly that each arose as a single hyperplastic muscle growth which, as it increased in size, displaced the remainder of the uterus in such a way that the latter formed an eccentric cap over one side of the tumor (Figs. 2 and 3). Except when the pyometra was extreme, all of the layers of the uterus were complete in this cap, including the endometrial glands (Fig. 4). The muscle layers on the tumor side of the lumen were lost in, and continuous with, the muscle fibers of the tumor. The serosa and the subserosal layers of connective tissue were stretched to form the capsule of the tumor. It was impossible to determine whether the tumor

FIGS. 1–7.—All of the tissues were sectioned at 8 μ and stained with Erlich's hematoxylin and triosin.

FIG. 1.—An extremely low power view of a cross section of the largest leiomyoma found in an endocrine imbalance female rat, showing the general characteristics of the tumor. The cavity is where pyometra had involved and distended the original lumen of the uterus. The thin wall is similar to that found around the large necrotic mass produced by pyometra. The other side is composed of tumor tissue. For comparison, a cross section of the uninvolved horn of the uterus at the same magnification as the tumor is shown in the small insert. ×2.5.

FIGS. 2 and 3.—Cross sections of medium sized tumors illustrating how the smooth muscle tumor forms in a localized area in the muscle layer. As the tumor grows, the serosa remains intact and the remainder of the uterus becomes stretched over the tumor as a relatively thin eccentric cap, shown at the extreme top of each photograph. The tumor tissue is slightly darker than the rest of the uterus so that the junction between them can be seen even at this magnification. The small cavity in the uterine tissue at the very top of each section is the uterine lumen. The ragged outline at the lower edge is due to the fact that these are not quite complete cross sections. ×8.

FIG. 4.—Photomicrograph of the stretched normal uterine tissue which forms a cap over the tumor. The uterine lumen, which may be seen extending diagonally across the photograph, has been elongated by the stretching of the uterus over the tumor. Note the relatively low columnar epithelial lining of the lumen, the normal uterine glands at the left of the lumen and the normal tunica propria. The muscle tissue of the tumor may be seen in the lower right hand corner of the photograph. ×110.

FIG. 5.—An area of a leiomyoma showing both smooth muscle and connective tissue in a mixed portion of the tumor. ×110.

FIG. 6.—A high power view of the smooth muscle which comprises the entire tumor when it is small. × 220.

FIG. 7.—A portion of the tunica propria of an uninvolved segment of the uterus, illustrating the chronic low estrogen effect. The uterine epithelium at the top of the picture is only slightly stimulated. The glands are well developed without hypertrophy of the epithelial cell lining. ×110.

started in the circular or in the longitudinal muscle layers, but it certainly must have involved both at an earlier stage than we have been able to observe.

The portions of the uterine horns not involved in the tumors showed some hypertrophy. If the mucosa was eroded at the tumor level it was usually almost completely absent throughout the remainder of the uterine horn. Conversely, if the endometrial epithelium was intact in the tumor region it was complete at other levels, but the tunica propria was thickened, and the uterine glands were often distended (Fig. 7). In all cases the epithelium was low columnar (Fig. 7) indicating that the estrogen level was relatively low. The tunica propria was thickened by increased amounts of collagenous tissue. Both circular and longitudinal muscle layers were hypertrophied, and the entire uterus was heavily vascularized.

DISCUSSION

The tumors in the rats' uteri described here are similar in all respects to the fibromyomas which occur so frequently in the human. Approximately 20 per cent of all women have fibromyomas. The incidence is much higher in unmarried women beyond middle life, but as the gynecologists point out, these figures are misleading since a great many of these tumors give no symptoms. Curtis (2) states that "the origin of myomatous tumors remains unexplained, although evidence is at hand which indicates that disturbed balance of the ovarian hormones, notably hyperestrinism, is an important etiologic factor." Earlier, Witherspoon (10) had suggested that there is a correlation between hyperestrinism and the occurrence of fibromyomas. Credence is given to this suggestion by the fact that not only have fibroids been produced in guinea pigs by prolonged treatment with estrogen (5) but they have been produced with ease in this animal with many kinds of estrogenic compounds (3, 4). This hypothesis is, however, far from being generally accepted.

Leiomyomas are extremely rare in rats, although Bullock and Curtis (1) have shown that they do occasionally occur. Therefore, it is felt that the fact that 4 of 16 constant estrus female rats which survived over 3 years developed leiomyomas would indicate that the endocrine imbalance imposed on these animals had increased the chance of the formation of such tumors. These animals could not, of course, become pregnant, which fact places them in a different category from the females usually found in a colony and more nearly likens them to the unmarried group in the human. It may be that the conditions of pregnancy, which alter the relatively short cyclic changes in the uterus, have in themselves an inhibitory effect on the production of leiomyomas. Multiple leiomyomas have not previously been described in the rat, a fact commented on by Bullock and Curtis (1). It has been shown in the present investigation, however, that multiple myomas of the uterus occur in rats just as they do in humans.

The endocrine imbalance in these rats keeps the uterus under the influence of a chronic low level of estrogen which allows pyometra to develop in almost all of the animals. This is undoubtedly due to the continuous exposure of the uterus to the bacterial flora of the vagina (9). It is felt that when the animals are able to survive the pyometra until old age, the chronic stimulation of the smooth muscle by the estrogen greatly enhances the chances of tumor formation. The fact that the pyometra prevents prolonged survival, together with the fact that leiomyomas are rare in the rat, makes it extremely difficult to analyze the factors responsible for the tumors described here. It is doubted that the administration of exogenous hormones would be as successful in stimulating the formation of tumors while avoiding the lethal effects of pyometra as were the endogenous hormones produced under this endocrine imbalance.

SUMMARY

Of 185 endocrine imbalance female rats, 16 survived over 3 years. Leiomyomas were present in 4 of these 16 animals. One animal had multiple tumors; 2 were located in the middle and lower third of the right cornu, and a third was near the cervical end of the left cornu. The other 3 animals had single tumors. In one of these the tumor was located in the right cornu. In the other 2 it was in the left cornu. It is believed that the chronic estrogen stimulation resulting from the endocrine imbalance increases the chance of smooth muscle tumor formation.

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Transplantable Mouse Lymphosarcoma T 86157 (MB)* Studied *in vivo*, *in vitro* and at Autopsy

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INTRODUCTION

Where exactly round-cell sarcoma, lymphosarcoma, lymphosarcomatosis, leukosarcomatosis, and aleukemic and leukemic lymphadenosis should stand in pathology is still debatable (5, 7, 10, 11, 12, 13, 18). The answer to this question touches on what cells eventually are the vectors of malignancy. It is for this reason that it was thought desirable to submit a number of these tumors to a closer scrutiny. This has been done with two mouse tumors, both anatomically and by means of the tissue culture method. In this paper we will describe the first of these tumors.

SPONTANEOUS TUMOR T 86157

This tumor originated in a female mouse (86157), the mother of which was a F_2 hybrid: ♀ F_1dB × ♂ F_1B, and the father a strain B mouse.[1] When killed on Feb. 19th, 1935, mouse 86157 was 286 days old. She looked healthy but all external lymph nodes were enlarged: in the right jaw they were 21 × 13 × 12 mm., left jaw 7 mm., right axilla 10 mm., left axilla 9 mm., right groin 11 mm., left groin 9 mm. The thymus and spleen were somewhat enlarged; the liver was normal in size. The whole mesentery was changed into a cylindrical tumor mass of 30 × 7 mm. The follicles of the small intestine were swollen. The cut surface of all lymph nodes was white and semi-transparent.

On microscopic examination the lymph nodes appeared to be mainly composed of large, closely packed, round or polygonal cells containing large nuclei and scanty cytoplasm. (Fig. 1.) The nuclear membrane was conspicuous, the chromatin structure coarse; one or two nucleoli were in evidence. Both nuclei and cytoplasm were strongly basophilic. There were many mitoses. The stroma consisted of a number of thinly walled vessels from which extended tiny reticulum fibers.

Small groups of the cells, described above, were found in the liver capillaries; larger groups were situated in the periportal spaces, but massive infiltration of the liver as will be described in our transplantation experiments, was seen nowhere.

The structure of the spleen was normal. Its pulp contained many myeloid elements. The follicles were not enlarged; they contained a few of the large cells described above.

In the small intestine cells of the same kind were found in great numbers in the follicles, which penetrated into the subserosa.

In the lungs no abnormal cells were found.

The blood of this mouse had not been examined. In the larger vessels the white cells did not seem to be increased in number.

Epicrisis: in mouse 86157 a tumor-like growth of pathologic cells of a lymphoblastic character existed in the lymph nodes. Not all lymph nodes were affected to the same degree. In addition there was a discrete but distinct dissemination of these cells, many of them in mitosis, in other organs. These findings suggested the diagnoses of lymphosarcoma, round-cell sarcoma or aleukemic lymphadenosis. In our opinion we are justified in speaking here of a lymphosarcoma developing into leukosarcomatosis.

TRANSPLANTATION STUDIES OF TUMOR MB

Minced tumor in Tyrode's solution taken from the left inguinal lymph node of mouse 86157 was injected subcutaneously into 6 F_1Bd and F_1dB females. Transplantation was successful in all. One mouse died 56 and another 67 days after inoculation; four were killed after 26, 27, 49, and 68 days respectively. The tumor of the mouse killed on the twenty-sixth day was grafted into 6 F_1 hybrids. Since then the transplantations have been repeated regularly into F_1 hybrids. The time between the

* The spontaneous tumor which originated in mouse 86157 was called tumor T 86157; the tumor which developed in other mice after grafting with tumor T 86157 was called tumor MB. In earlier papers both names were used, often as synonyms.

† The latter for the hematology.

[1] d = dilute brown of Murray-Little. B = C57 black, Little. F_1dB = F_1d ♀ × B ♂.

transplantations became progressively shorter. After 1 year the tumor had to be grafted every 2 weeks; after 2 years every 10 days. Twelve years after the first transplantation the tumor had to be grafted every 7 or 8 days as death occurred from 11 to 14 days after inoculation. Except for growth rate this tumor maintained its original character.

In 1940, 172 transplantations had been carried out in altogether 517 mice. In the 501 F_1dB and F_1Bd hybrids all transplantations were successful. In 6 d and 4 B mice no tumors developed though they were inoculated twice. Out of 6 first-backcross mice from F_1Bd to B, 3 developed a tumor after inoculation and 3, although they were inoculated several times, did not. From this we may conclude that, for its growth, tumor MB needs B-factors which are lacking in d mice, as well as d-factors lacking in B mice. As the tumor grew in 3 out of the 6 backcross-to-B-hybrids it seems probable that only one growth factor, being present in d but lacking in B, is needed. (We are fully aware of the fact that the number of mice is far too small for a valid analysis of the factors necessary for growth. Nevertheless it seems highly probable that this tumor genetically behaves in the same way as the other transplantable tumors, as regards transplantability.)

At the present moment tumor MB has been transplanted 421 times with 100 per cent takes in F_1Bd hybrids. For several months, in the fall of 1945, we were forced to graft each time into 1 mouse only, because of shortage of mice caused by war circumstances. During this period we allowed our mice to live as long as possible to save animals. Once we had to transplant a tumor from a mouse which died earlier than was expected and yet this transplantation succeeded: tumor MB is what is sometimes unjustly called a "virulent" tumor.

ETIOLOGY

These studies were made in order to determine whether round-cell sarcoma MB is a so-called viral tumor. The tumors used for these experiments were allowed to develop for 6 to 8 days after grafting. Necrotic tissue was never used. As test animals only F_1Bd and F_1dB mice, being 100 per cent susceptible, were used. All work was done under aseptic precautions. Only some of the experiments, all of which gave similar results, will be described below.

Experiment 1. Tumor tissue was excised and ground with sand in a few drops of Tyrode's solution. An equal amount of Tyrode's was then added and the mixture was centrifuged at 3000 rpm for 10 to 15 minutes. The supernatant fluid was passed under vacuum through a Seitz filter (size 3). From 0.2 to 0.5 cc. of the various filtrates were injected subcutaneously into 18 mice. As none of these developed a tumor, the filtrate could not have contained an active tumor-inducing virus.

Experiment 2. It seemed possible that, if a virus had been present, it might have been absorbed or might have been unable to pass the fine pores of the filter. Besides it might have been destroyed by oxidation or autolysis during the course of preparation. Therefore further experiments were carried out with the assistance of Dr. H. G. Frenkel, Dr. G. M. van Waveren and Dr. L. W. Janssen of the "Veeartsenijkundig Instituut" at Amsterdam. As it seemed important to determine the moment at which the suspension or the fluid lost its tumor-producing power during the experiment, we injected material selected at different stages of the preparation of the filtrate.

Repeatedly tumor tissue was quickly frozen in a Dewar jar containing solid CO_2 and 96 per cent alcohol, and then thawed out again, in an attempt to kill the cells and to liberate a possible virus. While still frozen it was ground in a mortar. In order to obtain a finer suspension it was necessary to thaw out the preparation again and to grind it when unfrozen. Six cc. of Tyrode's solution were then added and the mixture was centrifuged for 10 minutes at 1500 rpm. The sediment was injected into 2 mice. No tumors developed.

Since we know that a very small number of washed tumor cells are capable of producing a tumor (8), we may conclude that by this treatment all tumor cells were killed, as no tumor occurred in any of the injected mice.

Experiment 3. This experiment was carried out at low temperature in an attempt to prevent oxidation and autolysis. Immediately after removal the tumor tissue was frozen in the refrigerator at $-15°$ C. After a few drops of Tyrode's solution had been added, the frozen tissue was ground with sand in a cooled mortar, and then divided into 2 portions, A and B.

After portion A had been frozen for 5 hours in a Dewar jar which contained solid CO_2 and 96 per cent alcohol, it was thawed out. After 0.75 cc. of Tyrode's solution of pH 7.4 were added to 0.5 cc. of this preparation, the emulsion so obtained was centrifuged at 3000 rpm in an ice-cooled tube for 5 minutes. The supernatant fluid was immediately injected into 2 mice, each of which received 0.2 cc. One of these mice was injected intraperitoneally and the other subcutaneously. The sediment was minced with scissors in iced Tyrode's solution for 2 minutes and, while mixed with sand, it was injected into 3 mice, each of which received 0.2 cc. Of these mice 2 were injected subcutaneously and

1 was injected intraperitoneally. None of the 5 mice injected developed a tumor.

Portion B was put into a Dewar jar for 10 minutes. Afterward it was dehydrated at −15° C. *in vacuo*. Two days later, when dehydration was complete, 2.2 cc. of cooled Tyrode's solution were added to and thoroughly mixed with 1 cc. of the dehydrated powder, consisting of both tissue and sand. After having been centrifuged at 3000 rpm in an iced tube for 3 minutes, 0.5 cc. of the supernatant fluid were injected into each of 3 mice, of which 2 were injected subcutaneously and 1 was injected intraperitoneally. The rest of the supernatant fluid was carefully mixed with the upper layer of the sediment. Of this mixture 0.5 cc. was injected subcutaneously into 1 mouse, and 0.2 cc. was injected intraperitoneally into another mouse. The rest of the sediment was mixed with 0.5 cc. of Tyrode's solution and of this mixture 0.3 cc. was injected intraperitoneally into 1 mouse. None of the 6 mice injected developed a tumor. Since by injection of these sediments no tumors were produced, it was evident that all tumor cells were killed, as in experiment 2.

Experiment 4. Engelbreth-Holm (4) described a leukemia in mice which was transplantable by means of cell-free fluids, which, in order to prevent oxidation of the virus, were prepared in a nitrogen atmosphere. Our fourth experiment was made to ascertain whether it would be possible to obtain positive results with injections of cell-free fluids of lymphosarcoma MB by following Holm's technique. Holm used the supernatant fluid after centrifuging, and not the filtrate. As we knew that a few cells of our sarcoma were capable of producing a tumor after inoculation, it was essential to be certain that the supernatant fluid was absolutely cell-free. Accordingly we not only rigidly followed Holm's technique, but we moreover also passed the supernatant fluid through a no. 45 Elford filter. In this experiment all manipulations were carried out in a chamber perfused with nitrogen. All tubes and apparatus were filled with nitrogen before use. Ice-cooled tools were used where possible. The tissue, however, was not frozen.

The tumor-bearing mice were killed within 3 minutes by intraperitoneal injection of 1 cc. of 40 per cent potassiumferricyanide. The tumors were taken out as quickly as possible, minced with scissors, mixed with 0.5 cc. of a 0.2 per cent cysteine solution in Tyrode's, and ground with sand. Then 9.5 cc. of cysteine solution was added.

At various moments in these experiments a few drops of 1 per cent cobalt sulphate solution were added. The emulsions were centrifuged 1 to 3 times. In 2 of the experiments, the supernatant fluid was filtered through an Elford filter no. 45 after the last centrifugation. At various moments of each experiment samples were injected into mice, to check whether the tumor-producing agent was still present.

Table 1 shows the course and the results of 4 separate experiments a to d. We will describe experiment d as an example. After the tumor tissue was ground and the cysteine solution added, the emulsion was centrifuged for 10 minutes at 3000 rpm (= Centr. I). Three drops of $CoSO_4$ were added and the emulsion was centrifuged for the second time (= Centr. II). The supernatant fluid was then passed under pressure through an Elford filter no. 45. The outcome of this experiment was that 2 mice injected with sediment I (the sediment after the first centrifugation) produced tumors; both mice injected with sediment II failed to produce a tumor. Seven mice injected with the fluid which had passed through the Elford filter failed to produce a tumor also.

TABLE 1

RESULTS OBTAINED IN CENTRIFUGATION EXPERIMENTS 4A, 4B, 4C, AND 4D. THE EXPLANATION OF THIS TABLE IS GIVEN IN THE TEXT

Expt. no.	$CoSO_4$ (in drops)	Centr. no. I. Min.	$CoSO_4$ (in drops)	Centr. no. II. Min.	$CoSO_4$ (in drops)	Centr. no. III. Min.	Injected with sediment I	Injected with sediment II	Injected with supernatant fluid II	Injected with supernatant fluid III
4a		14		15	3	10	2 pos.			8 neg.
4b	5	15		15			2 pos.		8 neg.	
4c		10	3	10			2 neg.	2 neg.		
4d		10	3	10			2 pos.	2 neg.		

In 3 out of these 4 experiments tumors were produced by injection of the sediment of the emulsion which was centrifuged only once. The negative result in experiment c probably has to be attributed to the fact that the quantity of the cast was exceedingly small. Therefore: the tumor cells were not destroyed at this phase of the preparation. In all 4 experiments the tumor-producing power was lost after the second centrifugation.

Conclusion. The evidence obtained in all experiments indicates that for successful transmission of round-cell sarcoma MB living cells are indispensable. It has not been possible to gather

evidence of the existence of a tumor-inducing virus. If such a virus were to exist, it would have been very labile.

(The fact that in transplantable tumor MB no tumor-inducing virus was found, does not exclude the spontaneous tumor T 86157 having been induced by such a virus.)

PATHOLOGY OF MICE GRAFTED WITH TUMOR MB

In 1940 and 1941 fifty F_1Bd females, belonging to 4 different groups, which were grafted with tumor MB, were submitted to a close scrutiny. They were all 10 to 17 months old. In the fourth group the grafting was carried out with a line of the tumor which had been grown *in vitro* for 27 days between the 190th and 191st transplantation. We will come back to this point in another paper. All inoculations were carried out with tumor emulsion injected subcutaneously in the region of the right shoulder. From the first day onwards the blood picture was studied and mice were killed for gross and microscopic examination. Except for slight variations in the growth rate of the tumors—probably due to differences in the amount of tumor emulsion injected—neither clinical nor anatomical differences between mice of these 4 groups were found.

Hematology.—Blood was obtained by making an incision in the ventral or lateral vein of the tail of an etherized mouse. This method provides sufficient blood for a leukocyte count and for coverslip smears. Care was taken to avoid pressure on the tail. After the blood samples were taken, the wound was treated with iodine. No mice were lost and no infections were noted. The smears were stained with May-Grünwald-Giemsa. In every case 200 cells were counted.

In making differential counts we attempted to follow Petri's classification (15, 16) of normal laboratory mouse leukocytes. According to Petri, in the more immature neutrophils the nucleus is a smooth thick ring with but a small opening and fine chromatin. As the cells mature, the ring becomes thinner, the opening larger, and the chromatin coarser. Finally the ring appears as a lobed structure, the lobes often being connected by very thin filaments. Cells of all these types, as described by Petri, were found in our smears.

However, in agreement with Barnes and Sisman (1) and contrary to Petri we noticed that in the blood of healthy and of tumor-bearing mice almost all the neutrophils contain open nuclei instead of closed rings. We often noticed neutrophils with sausage shaped nuclei. The cytoplasm and the chromatin of these cells showed the same structure as that shown by the polymorphonuclears. The cells as described above looked so much like human metamyelocytes that we decided to classify them as immature polymorphonuclears, calling them "stab"-cells. They were notably increased in number shortly before death, when there was a marked leukocytosis. In addition, typical metamyelocytes and myelocytes were noticed.

We also always noticed a few neutrophils with ring-shaped nuclei with mature chromatin structure and with mature cytoplasm. This finding suggests the possibility of a developmental series different from the one just described. Their number was so insignificant however, that we felt justified in reckoning these as mature neutrophils.

We agree with Petri's classification of the mononuclear cells. Most of the lymphocytes were small cells with a round nucleus and scanty basophilic cytoplasm. Larger lymphocytes were noticed, with more cytoplasm and a more or less indented nucleus. Petri described some cells, the nuclei of which were divided into equal parts, bound together by a thin thread. Large lymphocytes cannot always be distinguished easily from monocytes. The monocytes (Petri's "large mononuclear cells") of the mouse have pale basophilic cytoplasm, in which granules neither can be seen, nor demonstrated with the peroxidase test. All transitions from round to decidedly lobulated nuclei were seen.

In the later stages of the tumor growth many leucocytes showed toxic granulation and cytoplasmic vacuolization; the nuclear chromatin became abnormally coarse. Similar changes are often observed in human pathology. Owing to these changes it was not always easy to classify the cells in the course of the last days before death, especially since at that period immature cells with similar toxic changes appear in the blood stream. Even in the blood of normal mice we occasionally found cells which we were unable to classify. (Petri's "unbestimmbare Zellformen.")

The average leukocytic count for 9 normal F_1Bd mice was 4750 cells (2500 to 8100). For 84 normal mice Petri found an average of 6172 leukocytes. Our differential counts were comparable with those of Petri: 0 to 3 per cent eosinophils (Petri 2.3 per cent); 0 to 1 per cent "Stab"-cells (Petri 0 per cent); 20 to 30 per cent polymorphonuclears (Petri 26.3 per cent); 65 to 80 per cent lymphocytes (Petri 65.3 per cent); 3 to 6 per cent monocytes (Petri 6.5 per cent).

In the blood of the normal mouse we noticed, in accordance with Petri, pronounced polychromasia, as compared to human blood. Polychromasia was significantly increased in mice which had been bled 2 or 3 times. Evidently many new red cells were formed after bleeding and immature red cells appeared in the peripheral blood.

During the first 4 days after grafting, the number of leukocytes and the differential count were normal. (A remarkable exception to this rule, consisting in a transient lymphopenia in a number of mice, will be dealt with by one of us in another paper,[1a] in which it will be demonstrated that this lymphopenia was caused by circumstances quite independent of tumor growth itself. The blood findings for group 2 are given in Table 2.

During the development of the tumor the following changes in the peripheral blood took place:

1. On the sixth to eighth days an increasing leuko-

[1a] W. M. de Bruyn. Cancer Research. In press.

cytosis appeared. In group 1, 12,400, 13,300 and 15,700 white cells were counted on the tenth day. In group 2, in which the tumor grew most rapidly, a leukocytosis of 11,670 and 14,325 was noted on the eighth day. In group 3, there was no evident leukocytosis. The leukocytosis was not caused by the presence in the blood of great numbers of tumor cells; on the tenth day tumor cells, which, when present, can be easily recognized in the blood-smear, were absent or present only in insignificant numbers. The findings in group 1 clearly demonstrated that the leukocytosis was partly due to the increase of the monocytes: they were 20 and 25 per cent on the eighth day, 26, 32 and 38 per cent on the tenth day, and 24.5 and 28.5 per cent on the twelfth day.

2. The monocytic phase is followed by a shift to the left, similar to the one seen in serious infections in man: myelocytes and metamyelocytes, metamyelocytes with nuclear chromatin structure already resembling that of polymorphonuclears, "Stab"-cells and polymorphonuclears with basophilic cytoplasm, the maturation of the nucleus and cytoplasm not always going hand in hand. The same cells were seen in bone marrow smears.

3. Tumor cells began to appear in the peripheral blood for the first time on the tenth day. The final phase was characterized by large numbers of tumor cells and a very high leukocytic count. In the last days before death the number of tumor cells continually increased. Due to these rapid changes, great variations in the leukocytic count and in the percentages of the different types of cells were found.

4. A relative lymphopenia existed in the final stages; the absolute numbers of the lymphocytes however were increased.

5. Shortly before death polychromasia was very strong indeed.

Anatomy.—It is often difficult to differentiate tumor cells from slightly abnormal cells, especially when they are located in the spleen. If these cells are lying in groups however, there is less difficulty. The study of the development of the tumor in a series of mice helped us in setting more clear cut criteria for the classification of these cells. In recording our results we speak of tumor cells only when the identification is certain.

Before the sixth day after grafting no signs of generalization were ever found. The first mouse fully described will be a mouse which was killed 6 days after grafting. The mice described belong to different groups.

Mouse 50055. The blood was examined 6 days after grafting. The mouse was killed directly afterwards. Blood: 8950 white cells per cmm.: lymphocytes 56 per cent, "Stab" cells ½ per cent, neutrophilic polymorphonuclears 24 per cent, eosinophils 1 per cent, monocytes 16 per cent, unclassified cells 2½ per cent, no tumor cells.

Autopsy findings: Subcutaneous tumor 20 × 5 mm.; lymph nodes and liver of normal size; spleen 22 × 5 mm.

Histology:

Subcutaneous tumor (Fig. 2): The picture of this tumor closely duplicates the picture given by the lymph nodes of the original mouse 86157, the only difference being the far greater number of mitoses in the transplant tumor. (As in the later stages of the disease the picture of the transplant tumors, save for slight necrosis, was always the same, it will not be described in the other mice.)

Liver: One to 3 tumor cells in only a few capillaries; some cells were in mitosis. No tumor cells in the walls of the larger vessels.

Spleen: Normal follicles, pulp hyperemic and slightly myeloid, no tumor cells.

Lymph node (left axilla): Partly myeloid, few solitary tumor cells in the capillaries.

Bone marrow, kidney, adrenal, lung, pancreas: in none of these organs tumor cells were found.

Mouse 50058. Examination of the blood 8 days after grafting. The mouse was killed directly afterwards. Blood: 11670 white cells per cmm.: lymphocytes 47½ per cent, neutrophilic polymorphonuclears 37 per cent, eosinophils 1 per cent, monocytes 5½ per cent, unclassified cells 9 per cent, no tumor cells.

Autopsy findings: Subcutaneous tumor 35 × 10 mm., right axillary and inguinal lymph nodes slightly en-

TABLE 2

BLOOD COUNTS IN ABSOLUTE NUMBERS IN THE MICE OF GROUP 2, AFTER GRAFTING. ON EACH OF THE DAYS INDICATED, THE BLOOD OF TWO MICE WAS EXAMINED. FOR THE DECREASE IN THE NUMBER OF LEUKOCYTES ON THE SECOND DAY, SEE TEXT. (NUMBERS IN ITALICS INDICATE PERCENTAGES.)

	1st day		2d day		4th day		6th day		8th day		10th day		12th day		13th day	
Mouse no.	50040	50042	50046	50048	50052	50053	50054	50055	50056	50058	50064	50066	50068	50070	50071	50072
Eosinophils	15	?	0	8	0	22	150	89	215	117	832	1076	801	730	0	886
	½	*1*	*0*	*1*	*0*	*1*	*2½*	*1*	*1½*	*1*	*4½*	*3*	*1*	*2*	*0*	*1*
Promyelocytes	0	0	0	0	0	0	0	0	0	0	0	0	0	1460	0	443
	0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*4*	*0*	*½*
Myelocytes	0	0	0	0	0	0	0	0	0	0	142	179	1604	1820	2420	5760
	0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*½*	*½*	*2*	*5*	*1½*	*6½*
Metamyelocytes	0	0	11	0	0	0	30	0	0	0	832	539	4020	1820	6460	3550
	0	*0*	*1*	*0*	*0*	*0*	*½*	*0*	*0*	*0*	*4½*	*1½*	*5*	*5*	*4*	*4*
Stab-cells	60	?	22	16	0	0	60	45	72	0	642	1257	4420	1820	5650	4440
	2	*1½*	*2*	*2*	*0*	*0*	*1*	*½*	*½*	*0*	*3½*	*3½*	*5½*	*5*	*3½*	*5*
Polymorphonuclears	795	?	910	632	2525	717	1900	2152	3440	4320	9110	14560	33750	16389	26620	36700
	26½	*25½*	*81*	*79*	*50*	*33*	*31½*	*24*	*24*	*37*	*49½*	*40½*	*42*	*45*	*16½*	*41½*
Lymphocytes	2070	?	148	128	1969	1348	3490	5010	8098	5543	5600	9510	16265	7100	16950	15530
	69	*70*	*13*	*16*	*39*	*62*	*58*	*56*	*56½*	*47½*	*30½*	*26½*	*20½*	*19½*	*10½*	*17½*
Monocytes	45	?	34	16	556	88	150	1430	2500	640	1108	8600	0	1639	0	0
	1½	*1½*	*3*	*2*	*11*	*4*	*2½*	*16*	*17½*	*5½*	*6*	*24*	*0*	*4½*	*0*	*0*
Unclassified cells	15	?	0	0	0	0	240	224	0	1050	184	179	0	0	0	0
	½	*½*	*0*	*0*	*0*	*0*	*4*	*2½*	*0*	*9*	*1*	*½*	*0*	*0*	*0*	*0*
Tumor cells	0	0	0	0	0	0	0	0	0	0	0	0	19290	3642	103500	21341
	0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*24*	*10*	*64*	*24*
Totals	3000	?	1125	800	5050	2175	6000	8950	14325	11670	18450	35900	80150	36420	161600	88650

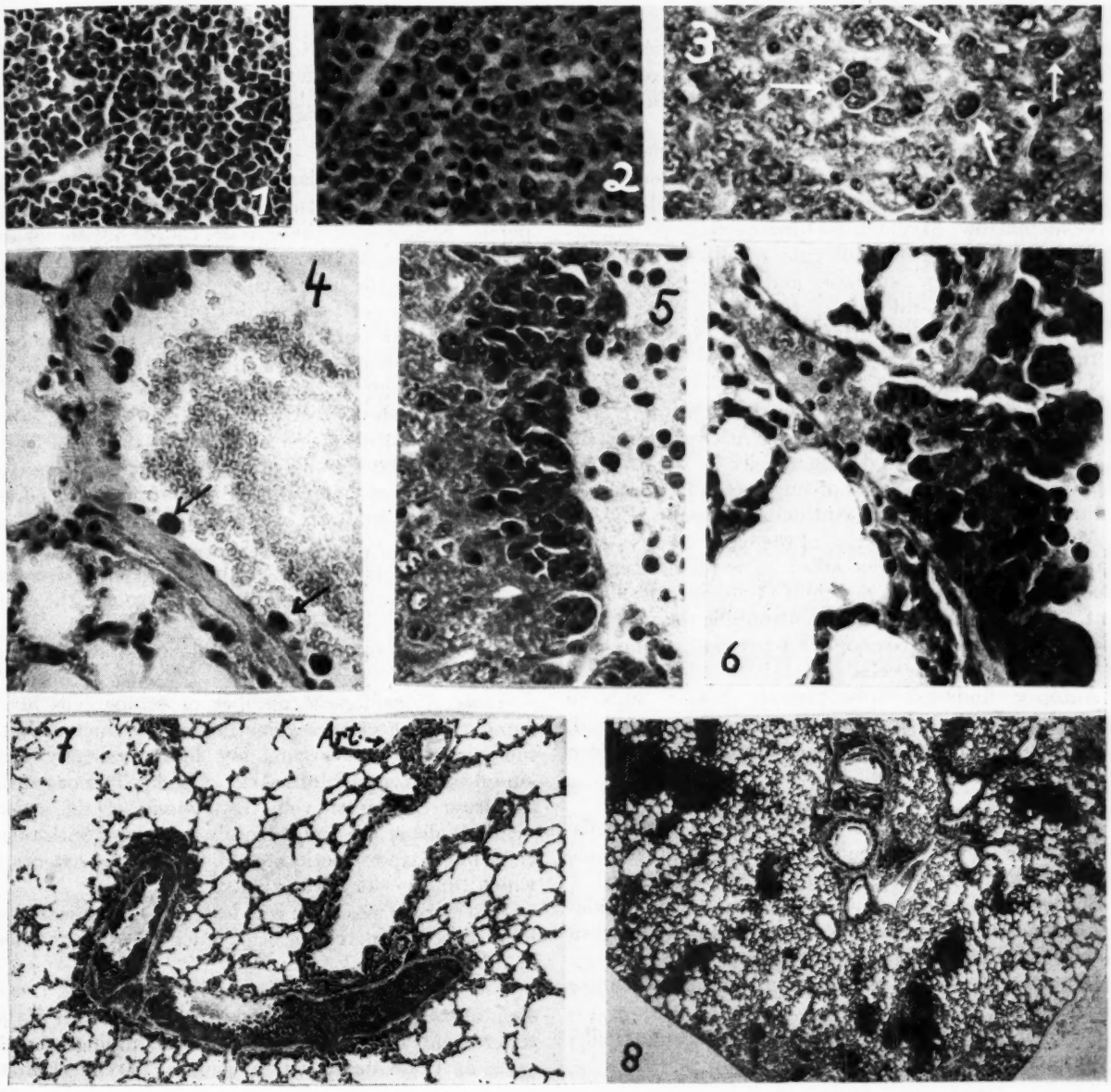


FIG. 1.—Section of spontaneous tumor T 86157. Mag. ×292.

FIG. 2.—Section of transplant tumor MB. Mag. ×350.

FIG. 3.—Eight days after grafting. Section of liver. Small groups of tumor cells in capillaries. Mag. ×350.

FIG. 4.—Eight days after grafting. Section of lung. Isolated tumor cells adhering to the intima of a larger vein. Mag. ×350.

FIG. 5.—Ten days after grafting. Section of liver. Wall of portal vein cuffed with tumor cells. Mag. ×350.

FIG. 6.—Ten days after grafting. Section of lung. Tumor mass penetrating from the lumen of a larger vein into a tributary vein. Mag. ×350.

FIG. 7.—Ten days after grafting. Section of lung. Lumen of a larger vein obstructed by tumor. Corresponding artery free from tumor. Mag. ×61.

FIG. 8.—Thirteen days after grafting. Section of lung. Most of the larger veins totally obstructed by tumor. Mag. ×23.

larged, other lymph nodes and liver of normal size, spleen 25 × 8 mm.

Histology:

Liver: In a few capillaries (Fig. 3) and around the larger portal veins small groups of tumor cells, numbering 10 to 50 cells in the latter. Many mitotic figures. Parts of the liver absolutely free from tumor cells.

Spleen: Small groups of tumor cells both in follicles and in pulp. Myeloid foci in the pulp.

Lymph node (left axilla): Exceedingly few tumor cells, partly in small groups. By far the greater part of this lymph node free from tumor.

Bone marrow: Myeloid, no tumor cells.

Kidney: Small groups of tumor cells with mitotic figures in only a few capillaries and venules.

Adrenal: Peripheral part of the cortex only at one place densely infiltrated with tumor cells. No tumor cells in the medulla.

Lung and pancreas: No tumor cells.

Mouse 50844 was also killed on the eighth day after grafting. Same findings as in mouse 50058 except for the lungs, in which scattered tumor cells in some of the capillaries and small groups of tumor cells in the lumen of the veins, adherent to the intima, were found (Fig. 4).

Mouse 50846. Examination of the blood 10 days after grafting. The mouse was killed directly afterwards. Blood: 16600 white cells per cmm.: lymphocytes 39 per cent, "Stab"-cells 1 per cent, neutrophilic polymorphonuclears 47 per cent, eosinophils 1 per cent, monocytes 12 per cent, no tumor cells.

Autopsy findings: Subcutaneous tumor 30 × 10 mm., right axillary lymph node somewhat enlarged, other lymph nodes and liver of normal size, spleen 17 × 5 mm.

Histology:

Liver: In most capillaries small groups of tumor cells. Walls of the portal veins cuffed with dense infiltrations of tumor cells (Fig. 5).

Spleen: Original structure of the organ still recognizable, although large groups of tumor cells are found in follicles and pulp. Pulp partly myeloid.

Lymph node (right axilla): Small groups of tumor cells.

Bone marrow: Myeloid. Many groups of tumor cells, occupying only a small part of the organ.

Kidney: Many small groups of tumor cells in the cortical capillaries; large infiltrations of tumor cells around the larger veins, isolated cells and groups of tumor cells adhering to the intima.

Adrenal: In the peripheral part of the cortex small groups of tumor cells.

Lung: Capillaries containing a moderate number of tumor cells. In most of the larger veins tumor growth has progressed so far that, save for a small opening in the centre, the lumen is oliterated (Fig. 7). The tumor has penetrated and totally obstructed the smaller tributary veins (Fig. 6). The walls of the larger veins are densely infiltrated with tumor cells, whereas the walls of the arteries and of the bronchi are free from tumor.

Pancreas: No tumor cells.

Mouse 50071. Examination of the blood 13 days after grafting. The mouse was killed directly afterwards. Blood: 161600 white cells per cmm.: lymphocytes 10½ per cent, myelocytes 1½ per cent, metamyelocytes 4 per cent, "Stab"-cells 3½ per cent, neutrophilic polymorphonuclears 16½ per cent, no monocytes, tumor cells 64 per cent.

Autopsy findings: Subcutaneous tumor 40×20 mm., right axillary gland 5 mm., other lymph nodes normal in size, considerable enlargement of the liver, spleen 30×10 mm.

Histology:

Liver: Most capillaries engorged with tumor cells. Dense infiltrations of tumor cells round the larger veins; partial occlusion of these veins caused by tumor cells pushing the intima inward. Far more than half of the cut surface of the liver occupied by tumor cells.

Spleen: Almost totally substituted by tumor cells, so much so, that only in a few places small groups of lymphocytes and of myeloid elements indicate where follicles and pulp were located.

Lymph node (left axilla): Half the lymph node is massively infiltrated with tumor; in the other half a number of isolated small groups of tumor cells is present.

Bone marrow: Much tumor in the smaller blood-vessels; massive infiltrations of tumor cells around the larger vessels.

Kidney: Much tumor in the smaller blood-vessels; massive infiltrations of tumor cells around the larger vessels.

Adrenal: Many groups of tumor cells both in cortex and medulla.

Lung: Considerable number of tumor cells in the capillaries. The larger veins mostly obstructed by tumor growth, small remaining lumina usually being closed by thrombus formation (Fig. 8). Tumor extending from the larger veins into many of the smaller veins. Walls of the veins densely infiltrated with tumor cells, in contradistinction to the walls of the arteries, in which tumor cells are lacking.

Pancreas: The organ was lost. No tumor cells were found in the pancreas of other mice killed on the same or following days.

The majority of our mice with transplanted tumors spontaneously died on the twelfth or thirteenth day after grafting. The gross and microscopic findings were the same as those described for mouse 50071, which we therefore may accept as giving the picture of the terminal phase of this disease.

Pathogenesis and development of the disease caused by transplantable tumor MB.—When a number of viable tumor cells are injected subcutaneously, a tumor develops as a purely local growth at the site of injection. Although it is probable that already during the first days following grafting some tumor cells may pass into the blood stream, the number of these cells is so small that they cannot be found microscopically. Their presence might have been demonstrated if we had made use of the bioassay method described by Fürth (7).

The grafted tumor rapidly increases in size by means of mitotic cell division. The surrounding

tissue is infiltrated, the walls of blood-vessels are invaded, and the chances for tumor cells to enter the blood stream increase. Six days after grafting isolated tumor cells and small clumps of tumor cells can be observed in various organs. Eight days after grafting their number has become much larger. In liver, kidney, and adrenal they are mostly situated in the capillaries. In the lung they are lying in the capillaries and in the lumen of the larger veins, adhering to the intima.

At that moment the tumor cells are widely dispersed throughout the body; yet considerable parts of all organs are still free from tumor, so that there is no diffuse generalization. Wherever there are tumor cells, a number of these are in state of mitosis. Cell divisions follow one another very rapidly and every metastasis soon spreads over the surrounding tissue, so that by superficial inspection it might look as if there were a so-called system disease. A closer examination however gives us the certainty that this impression is not in agreement with reality.

During the next few days there is a rapidly progressive infiltration with tumor cells, but not all organs are equally affected. Most susceptible are the liver and, to a somewhat lesser degree, the spleen. Less susceptible are lung, kidney, adrenal and bone marrow. The susceptibility of the lymph nodes is low and only those draining the area of the subcutaneous tumor show conspicuous tumor growth. The pancreas never contains tumor cells.

Shortly before death only small areas of bone marrow are left. Large parts of the liver are destroyed, mostly by pressure atrophy caused by infiltration with tumor cells, but also by anemic necrosis. The spleen is almost completely substituted by tumor. Most of the larger pulmonary veins are no longer passable for blood. The fact that these mice did not die much earlier in the course of their disease makes us believe that the tumor cells are almost or totally devoid of toxic effects.

A good correlation exists between the hematologic findings and the findings at autopsy. Apart from a transient lymphopenia in some of our groups, which had nothing to do with the tumors as such, no important blood changes were found during the first 8 days after grafting. This is in agreement with the fact that on the eighth day there was as yet no conspicuous growth of tumor in the bone marrow. In the later stages of the disease, simultaneously with extensive infiltration of tumor in the bone marrow, immature myeloid cells appeared in the blood stream. The fact that the lymph nodes were only slightly involved, explains why the number of lymphocytes was hardly increased and even shortly before death was no more than doubled. Since the origin of the monocytes is still debatable, we will not venture an explanation as to the fluctuations in the number of these cells.

Spontaneous tumors, which were more or less comparable with our tumor T 86157 (MB), developed in hundreds of mice in the Netherlands Cancer Institute, during the course of years. Some of these mice were killed before the tumor had become large. Then we only observed a localized tumor, as was the case in our mice a few days after grafting with MB tumor. Other mice with spontaneous tumors were killed after the disease had developed further; others died spontaneously. Apart from the absence of a transplant-tumor, the pathologic findings in these mice were in all respects identical with those found in our mice grafted with tumor MB. When a mouse dies from leukosarcomatosis with microscopic findings as seen in mice grafted with tumor MB, it seems justified to assume that the disease originated at one point and probably even in one cell, and that it had nothing to do with a so-called system disease, being on the contrary of a typical neoplastic nature.

IN VITRO STUDIES OF TRANSPLANTABLE TUMOR MB

In order to get vital information about the tumor cells, explants were cultivated. In 1938 the first results of this investigation were published (3). In cultures of tumor MB three types of cells were observed: 1. round cells resembling lymphoblasts, 2. mesenchymal cells, and 3. macrophages. Transitional forms between lymphoblast-like cells and mesenchymal cells were never noticed. It was believed that the lymphoblast-like cells probably were the malignant constituent, but at that time it was not possible to cultivate these cells successfully. In the present paper we describe how we succeeded in maintaining these cells in continuous culture.

Technique.—First we used the classic coverslip method, Mayer's modification of the coverslip method (14), and Carrel's flask method. Finally we adopted the roller tube method of Gey (9).

Tyrode's solution was prepared according to Fischer's description (6) and usually was mixed with serum before use. The pH of the culture medium was maintained between 6.8 and 7.2. Chicken plasma was obtained, without the use of heparin, from the carotid artery of an etherized young rooster. Serum was obtained from the aortic blood of an etherized 6 months old Wistar albino rat. Mouse and rabbit serum were derived from heart blood. In preparing the chicken-embryo "press juice" we followed Fischer's method. Mouse-embryo extract was prepared from embryos 0.5 cm. long,

which were chopped with scissors into fine pieces. An equal amount of Tyrode's solution was added. The mixture was centrifuged. The supernatant fluid was opalescent and almost colorless.

In order to obtain isolated cells, small pieces of tumor were explanted into roller tubes. The clot contained 2 parts of chicken plasma, 1 part of 50 per cent mouse-embryo extract in Tyrode's solution, and 1 part of 50 per cent chicken-embryo extract in Tyrode's solution. The fluid medium consisted of a mixture of 3 parts of Tyrode's solution and 1 part of rat serum. Twenty-four hours after explantation many lymphoblast-like cells were seen in the liquid phase, which was removed and centrifuged for half a minute at 500 rpm. After the supernatant fluid had been sucked off, the sediment containing the cells was transferred into another tube containing the fluid medium only. This washing of the cells was repeated several times, after which the cells were isolated with Schouten's micromanipulator.

Apart from minor modifications Schouten's method (17) was followed. A platinum loop was used for manipulating small groups of cells. A glass loop with a 9 to 12 μ lumen was employed for one-cell isolations. All procedures were carried out at room temperature (60° F.) in a 3 to 1 mixture of Tyrode's solution and rat serum. A typical experiment ran as follows:

a. The microscope was installed between the two needle-holding units.

b. A moist chamber was prepared.

c. A mica coverslip was coated with trileurine and then sterilized by flaming three times.

d. Six cell-free droplets of medium were placed separately on the mica and an additional droplet, containing free cells, was placed at a safe distance from the other drops.

e. The coverslip with the droplets was rapidly placed on the moist chamber.

f. A single loop full of cells was passed into the first cell-free droplet. Further dilution from droplet to droplet was then made until 1 or the desired number of cells was obtained in 1 of the droplets.

g. The coverslip was removed and the part containing the selected cell or cells was cut off with sterile scissors.

h. A culture of embryonic mesenchymal cells of a normal mouse was superimposed on the isolated lymphoblast-like cell which was lying in the droplet on the mica coverslip. One drop of a mixture composed of 50 per cent chicken plasma and 50 per cent embryonic extract was added. By this means we tried to avoid the isolated cell being washed away.

i. The culture was then handled as a coverslip culture and, after it had grown for 2 or 3 days, it was transferred into a roller tube in which it could be maintained for a long period.

The types of cells observed in vitro.—As mentioned above, cells of 3 types were noted in vitro: many round cells resembling lymphoblasts, increasing numbers of mesenchymal cells, and various numbers of large phagocytic cells (Fig. 9).

The round cells, which were larger than lymphocytes, contained a large nucleus with a few nucleoli. The chromatin structure of the nucleus was finer than that of the nucleus of a normal small lymphocyte. In the scanty cytoplasm no granules could be demonstrated by means of the Nägeli-Jørgensen benzidine-peroxidase (10) or the Schultze indophenol-blue-synthesis (15) tests. The cytoplasm was strongly basophilic and often contained small vacuoles, some of which could be stained with Sudan III. The cells phagocytosed neither lithium carmine nor trypan blue. They moved rapidly and, while moving, a short tail appeared at the posterior end, giving the cells a characteristic hand-mirror-like shape. At the anterior end short broad pseudopodia were present. The movement typically resembled that seen in lymphoblasts and lymphocytes and could be clearly demonstrated in motion pictures (Fig. 10). The sum total of these characteristics led us to consider these cells as being closely related to lymphoblasts. These cells made up the main part of the tumor explant. In media containing a large proportion of embryonal extract these cells died out rapidly.

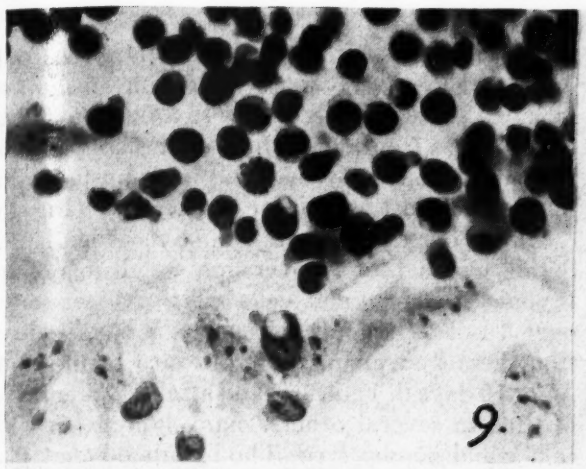
In the primary cultures, the mesenchymal cells appeared later than the lymphoblast-like cells. In media containing a large amount of embryonal extract, only the cells mentioned first, survived. Transitions between the lymphoblast-like cells and the mesenchymal cells were never seen.

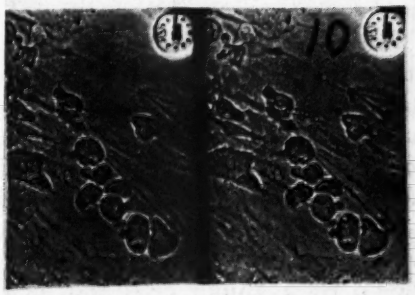
Macrophages were not always abundant in the cultures and usually disappeared after several transfers (Fig. 11).

The lymphoblast-like cell, the carrier of malignancy.—In order to find out which cells were the malignant ones, the isolated cells and cultures of the different types of cells had to be injected into susceptible mice. For subcutaneous and intraperitoneal injections of selected cells droplets were used containing one or several cells which were prepared by the method described above. Two-tenths cc. of Tyrode's solution were drawn into a sterilized syringe; a needle rinsed in Tyrode's solution was attached and the drop containing the selected cells was carefully sucked up. The entire amount was then injected.

In the case of whole cultures, colonies were put into the needle by means of a small stylet before it was attached to the syringe. Injections were made subcutaneously. After inoculation the needle was rinsed with Tyrode's solution and the rinsings were examined to make sure that all cultures really had been injected.

Isolated lymphoblast-like cells were found to produce tumors when injected intraperitoneally into a susceptible mouse. As few as 5 free lymphoblast-like cells could do this, death occurring with-





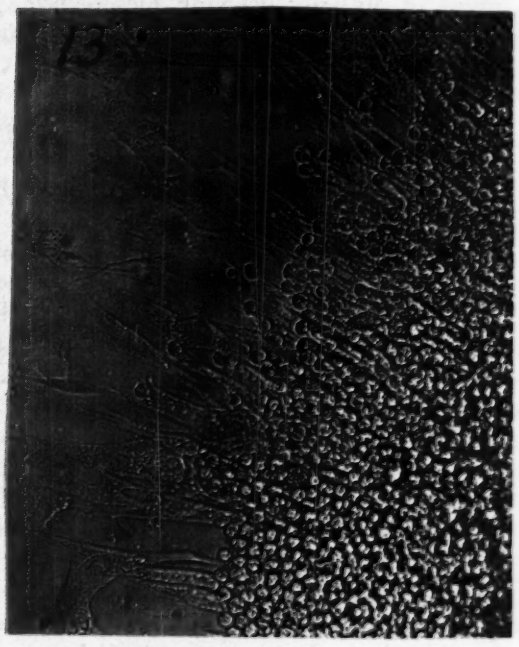


FIG. 9.—Fixed and stained 3 day old culture of lymphosarcoma MB. Large mesenchyme cells and many lymphoblast-like cells. Blunt "tails," large vacuoles, and mitoses are visible. Mag. ×570.

FIG. 10.—Living 5 weeks old culture of lymphosarcoma MB. Motion picture by Margaret Reed Lewis. Mesenchyme cells and lymphoblast-like cells. Blunt tails and vacuoles are visible. Mag. ×600.

FIG. 11.—Living culture of lymphosarcoma MB. Only mesenchyme cells are present. This culture was injected afterward and did not produce a tumor. Mag. ×15.5.

FIG. 12.—Horizontal section through a fixed and stained culture of lymphosarcoma MB, after 10 transfers. Darkly stained lymphoblast-like cells in the central part. Mag. ×325.

FIG. 13.—Living 1 day old culture, after 4 transfers, of the lymphoblast-like cells of lymphosarcoma MB, cultivated in the presence of mesenchyme cells of a normal mouse embryo. Mag. ×225.

in 16 days.[2] At least 70 cells were needed when subcutaneous injections were given. From this we conclude that the lymphoblast-like cell, being the cell predominating in sarcoma MB, is a malignant cell.

Forty mice were injected with cultures which, on gross appearance, contained only mesenchymal cells. Twenty-three developed a tumor while 17 did not. In 9 of the cases in which the inoculated mice grew a tumor, control subcultures were cut in horizontal slides. In the centre of all of these nine cultures, scattered and clumped lymphoblast-like cells were noted (Fig. 12). The occurrence of tumors in many of these mice therefore may be explained by assuming the presence of a few malignant lymphoblasts in what grossly appeared to be cultures of only mesenchymal cells.

Finally, we injected material from the peripheral areas of actively growing mesenchymal cells from cultures in which no lymphoblast-like cells could be discerned. Only one out of eight mice treated in this way developed a tumor.

In most cultures the macrophages disappeared at an early period. Since they were never found in abundance, experiments to determine whether they could produce a tumor or not, were not done.

From the results of the experiments described above we may conclude that, when injected into susceptible mice, actively growing mesenchymal cells of sarcoma MB alone do not produce a tumor. Consequently they cannot be looked upon as carriers of malignancy. These mesenchymal cells should be considered as belonging to the stroma of the tumor. The true tumor cells are the lymphoblast-like ones.

In vitro cultivation of the malignant lymphoblasts.—In order to cultivate the tumor cells in continuous culture it was necessary to use low concentrations of embryonal extract; moreover active mesenchymal cells had to be present (2).[3]

The tumor cells grew best in the following media: the clot containing 12.5 per cent mouse-embryo extract, 12.5 per cent chicken-embryo extract, 25 per cent Tyrode's solution, and 50 per cent chicken plasma; the fluid medium was composed of 75 per cent (or 78 per cent) of Tyrode's solution and 25 per cent (or 22 per cent) of rat serum.

Following the technique described above, we made cultures with 1 to 30 tumor cells put underneath cultures of embryonic mesenchymal cells. After 10 days large numbers of tumor cells were seen. It proved unnecessary to take the mesenchymal cells from mice which were susceptible for this tumor: mesenchymal cells from unsusceptible mice served just as well for this purpose (Fig. 13).

One of our experiments will be recorded here in detail. On each of 4 coverslips, 3 cultures of embryonic mesenchymal cells were put separately over 3 isolated tumor cells. After 2 days' cultivation these 4 coverslips were put into 1 roller tube. After 10 days in 1 culture, and after a longer period of time in several others, many lymphoblast-like cells could be observed. The likelihood that some of the 12 isolated tumor cells might have united to a little group of tumor cells, is so small, that we are fully entitled to believe that under proper environmental conditions 1 single tumor cell is capable of maintaining and reproducing itself in vitro.

As tumor cells without mesenchymal cells degenerated and soon died out, whereas it proved possible to cultivate cultures of both tumor cells and mesenchymal cells indefinitely, it seems probable that between these tumor cells and mesenchymal cells a kind of symbiosis exists.[4] Mice inoculated with these mixed cultures grown for 8 months, readily produced tumors.

Summary of the tissue culture investigation.—

1. In cultures of the lymphosarcoma MB, cells of 3 types were noticed: lymphoblast-like cells, mesenchymal cells, and macrophages.

2. The mesenchymal cells were the stroma. After injection with these cells, tumors never developed.

3. The lymphoblast-like cells, of which the tumors are chiefly composed, are the carriers of malignancy.

4. A tumor developed when a mouse was injected with no more than 5 lymphoblast-like cells.

5. These lymphoblast-like cells have been grown successfully *in vitro* for years.

6. *In vitro* the lymphoblast-like cells grew only in the presence of the mesenchymal cells. Under the conditions given, a kind of symbiosis apparently existed between the cells of these 2 types. The mesenchymal cells necessary for growth of the lymphoblast-like cells may be derived from both homologous and heterologous strains of mice.

CONCLUSION

By means of tissue culture and transplantation experiments and *in vivo* studies including gross and microscopic anatomy and hematology, it was

[2] Fürth and Kahn (8) produced tumors in mice by means of intravenous injections of one cell only.

[3] In the laboratory of Dr. Gey this strain of malignant lymphoblasts was maintained in continuous culture, in the presence of the original mesenchyme, for over 2½ years by Mrs. Margaret Gey. It was lost accidentally.

[4] Investigations on the reason why these tumor cells need mesenchymal cells for growth are being carried out at the present moment.

proved that mouse lymphosarcoma T 86157 (MB) is a real tumor. The malignant cell of this tumor is a pathologic lymphoblast. No filterable tumor-inducing virus could be detected.

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Human Sebum as Vehicle for Methylcholanthrene*

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Simpson, Carruthers, and Cramer (5) in 1945, demonstrated that methylcholanthrene when dissolved in anhydrous lanolin almost completely loses the power of inducing skin tumors in mice. It appeared desirable to Simpson, Carruthers, and Cramer to determine the reaction of the skin to the carcinogen dissolved in a medium that resembles sebum because, in their opinion, the sebaceous glands play an important role in experimental skin carcinogenesis. They chose lanolin because it represents the secretion of sebaceous glands of sheep; they assumed that the sebum of mice is similar to that of other species.

In connection with previous work (4) it appeared worthwhile to examine human sebum in the same way. To collect sufficient amounts of sebum from the skin surface of men is a difficult task. Large amounts of sebum, however, accumulate in the dermoid cysts of the ovary. These cysts are in part lined with skin epithelium, and large sebaceous glands secrete into the cavity of the cysts.

MATERIAL, METHODS AND RESULTS

The contents of dermoid cysts[1] were warmed slightly above body temperature; the oily yellow fluid could then easily be separated from the hairs and from the firmer whitish masses of desquamated squamous epithelium. Our assumption that this fluid represented chiefly the secretion of the large sebaceous glands was verified by determination of the lipid content in four specimens (100, 96.8, 99.5, and 99.6 per cent). This material was kept covered in the refrigerator; it was warmed before application until it was semifluid.

C 57 black mice (from Rockland Farms) were used. A 0.3 per cent solution of methylcholanthrene was applied to an unshaved area 1.5×1 cm., on the back, three times a week, for 14 weeks, using the same technique throughout.

The mice were divided into four groups, using benzene, lard filtrate, sebum from dermoid cysts, and lanolin, respectively, as vehicles. The mice in the lard filtrate group were 88 days old, at the beginning of the experiment, all the others were 37 days old. The results are summarized in Table 1. In the benzene group all of the 35 mice developed cancer as was to be expected. Fifty per cent of them had cancer after 16 weeks; they all were dead after 35 weeks. The results in the lard filtrate group were similar to those reported in the literature. Tumors appeared considerably later than in the benzene group, but after 29 weeks, 50 per cent of the animals had tumors, and after 1 year all of them. In the group of mice treated with methylcholanthrene in sebum from human dermoid cysts, the results were very similar to those in the lard filtrate group. In the lanolin group, however, none of the 20 surviving mice developed a tumor.

DISCUSSION

There is a significant difference in the carcinogenic response to methylcholanthrene when dissolved in sebum or lard filtrate on the one hand, and when dissolved in lanolin on the other. Since all 3 solvents are non-volatile and of similar consistency, this difference in carcinogenic response deserves consideration. It cannot, in our opinion, be explained as a mere dilution effect. As far as sebum is concerned it may be in line with the differences between different batches of lard or, for instance, the difference in carcinogenic response to solvents like sesame oil and tricaprylin. The use of lard filtrate and of dermoid sebum as vehicles led to delay of carcinogenic response when compared with benzene, while lanolin inhibited tumor formation in all surviving mice for a period of more than a year. The secretion of the sebaceous glands, which forms a film on the skin surface and on hairs, plays an important role in epidermal carcinogenesis. In the opinion of Suntzeff, Carruthers, and Cowdry (8) the sebaceous glands do not

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[1] The cyst contents were removed by hand from the ovarian dermoids. There was no extraction and no benzene was used.

merely represent portals of entry for the carcinogen but exert an influence upon the morphological and chemical condition of the epidermis. It is unknown how many factors may be involved in this influence and how far they would reinforce or counteract each other.

In human ovarian dermoids, which are tightly filled with sebum, carcinoma does occur. A survey of 1268 cases reported in the literature gave a percentage of 1.7 (3). In our own 328 cases, 4 carcinomas occurred. These tumors are squamous cell carcinomas, not of the ovary, but of the skin of the dermoid; they have all the ear-marks of skin cancer. It is unknown, whether or not the accumulated sebum contains a substance that induces these tumors, and it remains to be seen how far the same sebum, under the conditions of the mouse experiment, might be carcinogenic. Circumstances when we consider, for instance, the frequency of anal carcinoma in dogs. The dog has large, peculiarly differentiated anal glands whose secretion accumulates in pouches.

There was no significant difference in the delay of carcinogenesis between the sebum series and the lard filtrate series. Lanolin, however, inhibited tumor formation in all surviving mice as it did in the experiments of Simpson, Carruthers, and Cramer (5). We do not know why in the experiments of Berenblum and Schoental (1) lanolin was much less inhibitory. Differences in the animals used, and differences in the brands of lanolin may be considered. The mechanisms of retardation and inhibition of carcinogenic response by non-volatile solvents in general, and by lanolin especially, await elucidation. The retarding effect of liquid paraffin suggests a dilution effect (1) but the great

TABLE 1

SUMMARY OF RESULTS

Solvent	No. of mice	Effective total mice	No. of tumors	Time of first tumor (weeks)	No. of tumors (in weeks) 14	20	24	29	40	57
Benzene	35	35	35	7	13	27	33	35	35	35 (all)
Lard	30	26*	26	24	0	0	5	13	24	26 (all)
Sebum	35	28†	28	17	0	2	3	8	17	28 (all)
Lanolin	32	20‡	0		0	0	0	0	0	

* 3 died of infection, 1 of an accident.
† 5 died of infection, 1 was bitten to death, 1 died of unknown cause.
‡ 12 died of infection.

beyond our control prevent us at present from testing for carcinogenic, anticarcinogenic, or cocarcinogenic factors in human sebum. Numbers of other connected experiments that ought to be undertaken must, for the same reason, await a more propitious time. The study of sebum in relation to carcinogenesis, in our opinion, is of more than theoretical interest. The interaction between factors in the sebum and external potential carcinogens, when properly understood, might help in elucidating the genesis of some skin cancers, notably industrial cancers.[2]

The marked difference in carcinogenic response between human sebum (dermoid contents) and sheep sebum (lanolin) indicates possible species differences in the secretion of sebaceous glands. Simpson and Cramer (6) assumed that the sebum of mouse skin is similar to that of other species. Species differences as well as local differences in skin glands and their products suggest themselves differences between batches of lard and the difference between human sebum and lanolin, cannot be explained on such a basis. Weil-Malherbe and Dickens (9) have mentioned the possibility that the anticarcinogenic effects of animal fats might be caused by their content of phospholipids. The lanolin used by us, however, was free of phosphorus. It may be of more than passing interest that the sebum from dermoid cysts contained squalene but only negligible traces of "isocholesterol" (0.17, 0.02, and 0.07 per cent), using the method of Lederer and Kiun (2), while our lanolin was free of squalene and did contain "isocholesterol."

It is suggested elsewhere (7) that the triterpenoids of sebaceous materials may play some role in carcinogenesis or anticarcinogenesis.

[2] Almost all skin cysts which are commonly called dermoids or atheromas, in reality are epidermoid cysts. No glands, sebaceous or others, are found in their wall, they do not contain sebum but desquamated skin scales only. They, therefore, do not belong to the topic of this paper.

SUMMARY AND CONCLUSIONS

1. Human sebum, as a vehicle for methylcholanthrene, is similar to lard filtrate.

2. Sebaceous substances from different animal species seem to differ in relation to carcinogenesis.

3. The nature of the anticarcinogenic action of lanolin, so far, is unexplained.

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Effects of Phosphate Bond Energy on Nucleic Acid Stability in Tumor Homogenates*

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Recently in studies of anaerobic glycolysis in homogenates of a transplantable rat carcinoma (Flexner-Jobling) a medium was developed which is optimal for sustaining the phosphate bond energy (adenosine triphosphate) in the reaction mixtures (3). This should make it possible to study, in these homogenates, synthetic reactions which require energy. As a preliminary to these studies, an effort was made to determine whether nucleic acids were breaking down during incubation of the tumor homogenates and, if so, whether phosphate bond energy had any influence upon the reactions. Maintenance of phosphate bond energy was found to be correlated with maintenance of the nucleic acids.

EXPERIMENTAL

The tumors used were subcutaneous implants of Flexner-Jobling carcinoma in Sprague-Dawley rats of 100 to 200 gms. The implants were used at 8 to 12 days, when they were actively growing and free from any gross evidence of necrosis. The rats were killed by decapitation and tissues removed rapidly to small beakers of isotonic KCl imbedded in ice. After trimming away connective tissue, they were homogenized with Potter-Elvehjem homogenizers (5) in 9 volumes of cold isotonic (1.15 per cent) KCl.

The experiments were carried out in Warburg flasks at 37.6° C. with a 95 per cent nitrogen–5 per cent carbon dioxide atmosphere. Each flask contained the following additions, described as the "complete" medium; omissions were made where noted and water substituted: 0.30 ml. of 0.024M potassium phosphate (pH 7.4), 0.15 ml. of 0.5M potassium bicarbonate, 0.30 ml. of 0.4M nicotinamide, 0.15 ml. of 0.15M potassium pyruvate, 0.10 ml. of 0.01M adenosine triphosphate (ATP) potassium salt, 0.20 ml. of 0.003M diphosphopyridine nucleotide potassium salt, 0.20 ml. of 0.1M magnesium chloride, 0.10 ml. of 0.3M glucose, 0.15 ml. of 0.04M hexosediphosphate potassium salt, 0.15 ml. of 0.2M potassium fluoride, 0.30 ml. of 10 per cent homogenate of the tissue in isotonic potassium chloride,—and water to make the volume to 3.0 ml. Each flask was incubated the specified time, then stopped by addition of 0.25 ml. of 65 per cent trichloracetic acid from the sidearm. The contents were then transferred to a 13 × 100 mm. tube in each case, and the precipitated protein quantitatively transferred from the flask to the tube by means of two washes with small volumes of 5 per cent trichloracetic acid. The protein precipitate was centrifuged and the supernatant fluid discarded. The procedure for determining nucleic acids in these precipitates was that of Schneider (6), with modifications necessary for dealing with such small quantities of tissue (30 mg.). The procedure was as follows. Each precipitate was given two successive washes by suspending it in 3 ml. of 5 per cent trichloracetic acid, centrifuging and discarding the supernatant fluid each time. Then each was treated with 0.5 ml. of water plus 2.0 ml. of 95 per cent ethanol, centrifuged and the wash discarded. A second such wash was made with 2.0 ml. of ethanol to assure removal of the acid. The precipitate was now suspended in 2.0 ml. of 3:1 alcohol-ether and heated 3 minutes in a water-bath at 40° C. The precipitate was centrifuged and this treatment repeated twice. The alcohol-ether remaining was permitted to evaporate at 37° C. and the dry precipitate treated with 1 ml. of 5 per cent trichloracetic acid. The tube was now heated at 90° C. in a water-bath for 15 minutes. Aliquots of the acid were used to determine phosphorus and ribose by methods previously described (4).

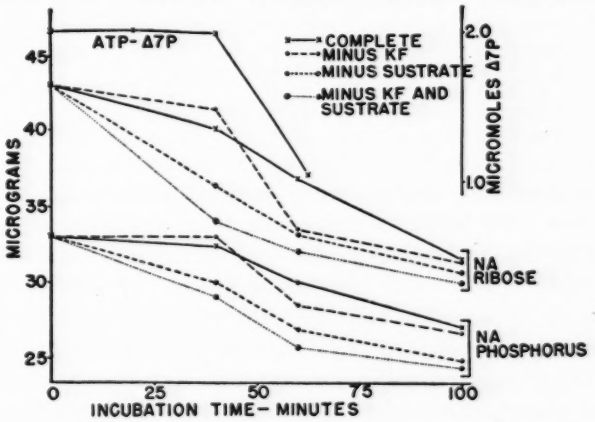

FIG. 1.—Stability of nucleic acids in tumor homogenates (30 mg. wet weight of tissue).

Some 7 experiments were carried out with the tumor tissue. The data are presented in Figure 1. Each experiment included duplicate flasks for each point given on

* This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

each of the curves except for the ATP curve, which was obtained in a separate experiment. The results of these experiments were averaged for the figure. There were variations in the amount of nucleic acid breakdown from one experiment to another but not in the relative positions of the curves.

Two experiments were carried out in a similar manner, with the same medium, using homogenates of brain. The averages of the results for these are given in Table 1.

DISCUSSION

It can be seen in Figure 1 that the ATP was maintained for 40 minutes in the tumor homogenates then declined rapidly. The decline results from the cessation of glycolytic activity due to exhaustion of substrate. The curve given is for a complete medium. Direct measurement of ATP was not made for the other conditions under which

TABLE 1

STABILITY OF NUCLEIC ACIDS IN BRAIN HOMOGENATES DURING ANAEROBIC GLYCOLYSIS

	Nucleic Acid Phosphorus per 30 mg. of Tissue			
Incubation time min.	Complete medium microgm.	Medium −fluoride microgm.	Medium −substrates microgm.	Medium −fluoride −substrates microgm.
0	11.3	11.3	11.3	11.3
40	10.6	10.5	10.0	9.8
60	10.1	10.0	10.0	9.5
100	10.6	10.6	10.5	9.9

nucleic acid studies were made, but it is obvious that ATP will decline rapidly from the start of incubation in the cases where substrate was omitted. Where ATP was maintained, the nucleic acids were apparently also maintained, and the loss of ATP is correlated with loss in the nucleic acids. There was not sufficient material for measurement of desoxyribonucleic acid, but from the measurements of total nucleic acid phosphorus and ribose, it is possible to calculate ribonucleic acid and desoxyribonucleic acid by difference. The initial levels found in these analyses agree well with results obtained by Schneider in his analyses of this same tumor (7). The decline appears to be approximately two-thirds due to ribonucleic acid, and one-third to desoxyribonucleic acid. When brain homogenates were studied, the decline in nucleic acid was negligible whether or not ATP was maintained. Brain appears to lack the potential for carrying out this reaction. Preliminary experiments with liver and kidney without a sustaining glycolytic system showed a loss of approximately 9 per cent of the nucleic acid phosphorus of liver in a 60 minute incubation and 18 per cent in kidney. These are then intermediate between brain with no breakdown, and tumor with approximately 23 per cent breakdown per 60 minutes.

The products of this reaction were not determined, but since they are acid-soluble, it is likely the reaction is a depolymerization. It is not possible to ascertain from these data whether the maintenance of nucleic acid by ATP represents prevention of breakdown or concomitant breakdown and resynthesis, nor is it apparent therefore whether the activity concerned is a reverse of normal nucleic acid synthesis. However some correlation seems to exist between ability to grow and possession of this activity on the part of the several tissues examined. Analytical data are available from the work of Greenstein (1, 2) on depolymerases of ribonucleic acid and desoxyribonucleic acid in a variety of tissues. There appears to be little difference between hepatoma and liver with respect to these enzymes. However, no data could be found for the assay of rat brain and Flexner-Jobling carcinoma for the depolymerases.

SUMMARY

Studies of the nucleic acid phosphorus and ribose in homogenates incubated at 37.6° C. demonstrated that when homogenates of Flexner-Jobling rat carcinoma were provided with ATP by means of an active anaerobic glycolysis, the nucleic acids were maintained. In the absence of a system to maintain ATP, the nucleic acid declined at a linear rate of approximately 23 per cent per hour. Brain homogenates maintained their nucleic acid even in the absence of ATP. Homogenates of liver and kidney gave intermediate rates of decomposition for nucleic acid (9 and 18 per cent respectively).

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Intraocular Transplantation of Malignant Lymphomas of the Mouse, Dog, and Man in Heterologous Species

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The transplantation of malignant human tumors in the eyes of guinea pigs and rabbits has been a helpful method for studying some neoplasms (4, 7, 10). Recently it has been advocated as a clinical-pathological technique to aid in the diagnosis of malignancy and degree of autonomy of tumors (11, 12). This method has, however, not been sufficiently well developed for use as a routine laboratory procedure. All types of tumors have not as yet been studied from this point of view, and the question is still unanswered why many neoplasms which are clinically highly malignant fail to establish a foothold in the anterior chamber and grow (5, 7, 14). That these tumors have not attained autonomy and therefore cannot grow after heterologous transplantation may indeed be an explanation for this phenomenon (12), but just what autonomy in this sense means to the welfare of the patient host and to a biological understanding of cancer is obscure. Several years ago experiments were begun in this laboratory to study malignant lymphomas by this method in the hope that an accurate earlier method of diagnosis of this difficult group of tumors might be developed, and that transplants which would be suitable for chemotherapeutic studies might be obtained. As yet no extensive study of malignant lymphomas has been reported although mention has been made in several instances that attempts at heterologous intraocular transplantation were unsuccessful (5, 12).

MATERIALS AND METHODS

Transplantation of tissue into the anterior ocular chamber was made by the method of Greene (6). As soon as possible after its removal from the donor, the tissue was inserted through a slit in the cornea. The pieces were trimmed aseptically to about 1 mm. or less in greatest dimension. Most tissues were introduced with 18 to 20 gauge needles; in a few instances firm rubbery tissues were inserted with a small forceps. The intraocular transplant was then moved into the inferior corneal angle by pressure from the outside with a glass rod. All instruments were sterilized before each operation in creosote solution and several washes in 70 per cent alcohol which was, in turn, permitted to evaporate. The conjunctivae were anesthetized with cocaine solution. In mice, the corneal slit was made with a sharp knife and an emulsion of tumor was injected into the anterior chamber, using a syringe and a 26 gauge needle with a short bevel. Our successful growth of a canine melanoblastoma through 2 generations of guinea pigs in other experiments showed that the techniques used in these experiments were adequate.

These experiments employed 70 young guinea pigs, averaging 500 grams in weight; 29 rabbits, averaging 2.5 kilograms in weight; 6 pure bred cocker spaniels, 8 weeks old; 20 strain Ak mice; and 20 strain CF_1 mice obtained from Carworth Farms. Pieces of tissue were usually implanted in both eyes of each guinea pig and rabbit. Transplants were made into only one eye of each animal in the first group of experiments and when dogs and mice were used. In all, transplants were studied in 111 eyes of guinea pigs, 56 eyes of rabbits, 2 eyes of dogs, and 20 eyes of mice. The eyes of the larger animals were examined on the average of 2 or 3 times a week for the first 4 to 8 weeks and then at irregular intervals for the next 6 months in guinea pigs and 4 months in rabbits and dogs. The eyes of the mice were examined every other day until all Ak mice were dead, and at increasingly long intervals after that for the next year and a half in the CF_1 mice. At these observations schematic drawings were recorded of the findings in each eye.

Eleven separate groups of experiments were made. Seven human malignant lymphomas were studied; 3 leukemias, 2 lymphosarcomas, and 2 cases of Hodgkin's disease. One suspected human leukemia turned out to be an acquired hemolytic anemia but the study of this tissue is included in this paper for comparative purposes. Three malignant lymphomas of animals were studied; canine malignant lymphoma, and 2 mouse leukemias. In 4 cases (Nos. 1, 2, 4, and 8) the clinical diagnoses were only provisional at the time of transplantation. It was hoped that this procedure would aid in reaching the correct diagnosis. In all cases the final diagnosis was made clinically and confirmed by pathological study of either biopsy or necropsy material.

Half of the tissue from each donor was studied histologically. In experiment 1, the lymph node obtained by biopsy from a boy aged 9 years contained blastic cells of unknown nature which were beginning to obscure the architecture of the node. In experiment 2, the lymph node obtained by biopsy from a boy 8 years old contained many hemocytoblasts distributed focally throughout the node. In experiment 3, femoral marrow

composed chiefly of leukemic myelogenous cells was obtained 1.5 hours after the death of the patient, a woman aged 65. In experiment 4, sternal marrow obtained by aspiration from a 13 year old boy was allowed to clot and the paler portions were used as transplants; large numbers of reticular cells were found among the cells present in these areas. An inguinal lymph node was obtained at necropsy in experiment 5 from a man 47 years old, about 2 hours after death; it was typical of far-advanced lymphosarcoma. The lymph nodes in experiments 6 and 7 were obtained by biopsy and were typical of untreated lymphogranulomatous Hodgkin's disease; the patients were women aged 26 and 32 years. In experiment 8 there was a marked reticular cell hyperplasia in the lymph node obtained by biopsy from a boy aged 8 years of age, a case which eventually proved to be one of acquired hemolytic anemia.

In order to follow histologically the fate of the human tissues transplanted into the anterior chamber of guinea pigs, the tumor-invaded lymph node in experiment 2 was also implanted intraocularly in both eyes of 6 additional guinea pigs, which were then killed 2, 4, 6, 8, and 14 days later for microscopic study of the implants. Occasionally rabbits and guinea pigs in other experiments were also killed for this purpose. All eyes were fixed in Zenker's formol solution, imbedded in celloidin after removal of the posterior chamber, sectioned, and stained with hematoxylin and eosin.

The canine tumor in experiment 9 was a malignant lymphoma causing great enlargement of all lymph nodes, thymus, spleen and liver of a pure bred cocker spaniel about 2 years old. In addition to guinea pigs and rabbits, used as in other experiments, an emulsion of tumor was injected into the eyes and under the skin of 2 pure bred cocker spaniel puppies and subcutaneously into 4 others of the same litter. On the day before injection, one of the dogs receiving the intraocular transplants and 2 of those receiving the tissue subcutaneously were given 130 r of total body x-radiation.

In experiment 10, a transplantable lymphatic leukemia originating in strain Ak mice was used. This tumor routinely takes successfully in all animals of this strain after subcutaneous injection. It kills in 14 days, with diffuse growth throughout all organs. Because this tumor metastasizes so rapidly and does not produce especially large local masses or lymph nodes, in experiment 11 another lymphatic leukemia of this strain of mice was used; it produces large local masses and greatly enlarged lymph nodes. It metastasizes widely, killing all Ak mice within 30 days. In addition to the usual guinea pigs and rabbits, this mouse tumor was also injected into the eyes of 10 young inbred Ak mice and 10 CF_1 strain mice and subcutaneously into a similar number of each strain.

RESULTS

Human leukemias and lymphomas.—The results of the first 8 groups of experiments, using human tissues, are summarized in Table 1. In these experiments no "takes" were obtained. No transplant was seen to increase in size during the 4 to 6 months of observation; all resorbed promptly. Typically, the appearance of the piece of tissue and of the anterior chamber followed a well-defined course. Twenty-four hours after transplantation the aqueous humor was hazy and the color of the piece of tissue was fading. By 3 days the conjunctiva at the limbus was hyperemic and the transplant was dull gray or yellow. By 7 days the piece was about one-half of its original size; occasionally it could no longer be found. The mild ophthalmitis was usually resolved by this time. By 14 days the pieces of tissue were about one-third their original size and ivory or light gray. The pieces decreased very little in size during the next 5 weeks although an occasional piece disappeared entirely. At the end of this time the tissue was minute and almost pure white. During the following months no changes were noted in its appearance. An occasional eye developed suppurative panophthalmitis. In most cases the transplants in the eyes of the guinea pigs resorbed faster than did those in the eyes of rabbits. In rabbits, during the first 48 hours, it was not unusual for a definite gray halo to develop around the transplant. Histologic study of such a halo (Fig. 6) showed that it was composed of fibrin. Most of the lymphocytes and granulocytes had died or disappeared from the transplant at this time (48 hours). The reaction and sequence of events appeared the same in the eyes regardless of whether one or both were implanted.

A histologic study of the fate of the transplanted tissue was made in experiment 2 in guinea pigs killed at 2, 4, 6, 8, and 14 days after the operation (Figs. 1 to 5). At 2 days the pieces were almost completely necrotic; they were eosinophilic and almost structureless. Most of the cells were dead. A few viable hemocytoblasts in the transplant were surviving in contact with the cornea in one eye (Fig. 1). In addition scattered endothelial and reticular cells were stainable. No viable cells were present in the transplant in the opposite eye; it was completely necrotic. At 4 days the transplants still contained minute foci of nuclear debris and they now contained many proliferating fibroblasts. These growing cells seemed to stem from the contiguous iris as no line of demarcation remained, and melanophores also were migrating into the transplants (Fig. 2). By 6 days, the fibroblastic tissue had produced collagenous fibers in the portion adjacent to the iris and was continuing to proliferate in the remaining part (Fig. 3). By 8 days, this connective tissue was almost completely differentiated; the transplant was composed of condensing collagenous connective tissue.

Lymphocytes and plasma cells were now present in the adjacent iris and in the margins of the transplant. They were probably of host origin since they were intermingled with pseudoeosinophilic leukocytes of the guinea pig (Fig. 4). After 14 days, the piece was a minute round body composed entirely of collagenous connective tissue (Fig. 5). mass was visible on the iris. By the fourth day this mass was seen as a tannish-white projection 1 mm. long, protruding from the angle of the iris; it had 3 tiny polypoid rounded masses at its free end. Four days later this projection was whiter and its margins were no longer distinct. By 12 days it was a stringy white structure; later it gradually con-

TABLE 1

SUMMARY OF THE INTRAOCULAR TRANSPLANTATION OF HUMAN LEUKEMIAS AND MALIGNANT LYMPHOMAS

No. of experiment	Provisional diagnosis	Material transplanted	No. of animals*	No. of eyes	No. of takes	Final diagnosis	Fate of donor
1	Leukemia?	Axillary lymph node	6 GP	66	0	Myeloblastic leukemia	Death 4 months later
2	Leukemia?	Inguinal lymph node	6 GP†	12	0	Stem cell leukemia	Death 3 months later
			3 R	6	0	leukemia	
3	Chronic myelogenous leukemia	Femoral marrow‡	6 GP	11	0	Chronic myelogenous leukemia	Dead at time of implant
			2 R	4	0		
4	Leukemia?	Sternal marrow	6 GP	11	0	Reticulum cell sarcoma	Death 1.5 months later
			3 R	5	0		
5	Lymphosarcoma	Inguinal lymph node‡	6 GP	11	0	Lymphosarcoma	Dead at time of implant
			4 R	8	0		
6	Hodgkin's disease	Inguinal lymph node	6 GP	12	0	Hodgkin's disease	Alive with disease 2.5 years later
			3 R	6	0		
7	Hodgkin's disease	Axillary lymph node	6 GP	12	0	Hodgkin's disease	Alive with disease 2.5 years later
			3 R	6	0		
8	Leukemia?	Axillary lymph node	4 GP	8	0	Acquired hemolytic anemia	Splenectomized; alive 2.5 years later
			2 R	4	0		

* GP = guinea pig; R = rabbit.

† Also transplanted into 6 additional guinea pigs for serial sacrifice (see Figures 1 to 5).

‡ Tissue obtained at necropsy and transplanted within 1.5 and 3.5 hours after death.

TABLE 2

SUMMARY OF INTRAOCULAR TRANSPLANTATION OF MALIGNANT LYMPHOMAS OF A DOG AND MICE IN GUINEA PIGS, RABBITS, DOGS AND MICE

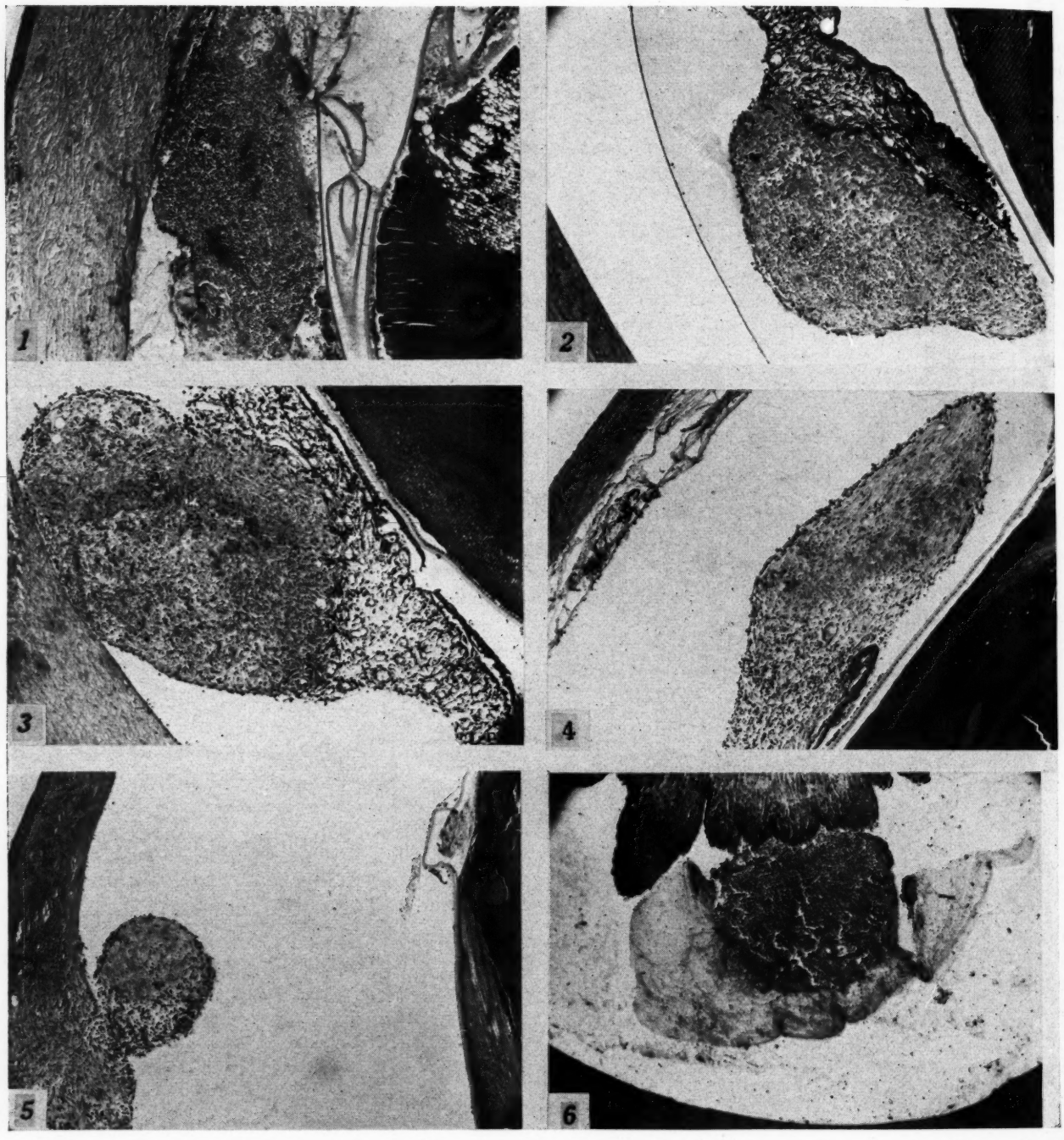
				Intraocular transplants				Subcutaneous transplants	
No. of experiment	Source of tumor	Type of lymphoma	Fate of donor	No. of animals	No. of eyes	No. of takes	No. of regressions	No. of animals	No. of takes
9	Dog	Malignant lymphoma	Death in 30 days	4 GP*	7	0			
				4 R	7	0			
				2 D	2		1	6 D	
10	Strain Ak mice	Lymphatic leukemia	Death in 14 days	6 GP	10	0			
				2 R	2	0			
11	Strain Ak mice	Lymphatic leukemia	Death in 30 days	6 GP	11	0			
				3 R	6	0			
				10 Ak	10	10	2†	10 Ak	10
				10 CF_1	10	10	9	10 CF_1	1

* GP = guines pigs; R = rabbits; D = pure bred cocker spaniels; Ak = strain Ak mice; CF_1 = strain CF_1 mice.

† These mice died with generalized leukemia after regression of the intraocular tumor.

There was no evidence in these sections that any cells of the donor survived over 48 hours.

Canine malignant lymphoma.—The canine malignant lymphoma did not take in the eyes of guinea pigs or of rabbits in experiment 9 (Table 2). The visible sequence of events resembled that of the human tissues. A transient take was apparently obtained in the eye of the cocker spaniel puppy that had previously been irradiated. Since an emulsion of lymphoma (rather than a piece) was injected into this eye, no tumor was seen for the first 2 days. On the third day a minute round densed. Six months later this dog developed a secondary glaucoma which, in spite of several trephine operations, persists at the present time, 2.5 years later. Although floating cells were seen by slit-lamp in the anterior chamber of the other dog no growth or glaucoma occurred. Local edematous masses were found at the sites of subcutaneous injection in the other dogs; they were slightly larger in the irradiated than in the nonirradiated animals. These areas of edema were gone in all dogs in 21 days. Three dogs died with distemper, verified by necropsy, at the age of 4 months. The



FIGS. 1–5.—These illustrate the histologic changes between 2 and 14 days in transplants of a lymph node from a case of stem cell leukemia (Experiment 2) in the anterior ocular chambers of guinea pigs. These photomicrographs are reduced from a magnification of ×120. The cornea is always to the left and the iris or lens is on the right. Some illustrations show artifacts.

FIG. 1.—At 2 days the transplant is partly necrotic.

FIG. 2.—At 4 days transplanted blast cells have disappeared and fibroblastic proliferation has begun.

FIG. 3.—At 6 days organization has proceeded and collagen is now visible near the iris.

FIG. 4.—At 8 days organization is almost complete.

FIG. 5.—At 14 days the organization is complete and the transplant is small.

FIG. 6.—Shows that the halo seen around the transplant in a rabbit was composed of fibrin. Unfortunately there is also present much staining artifact. Reduced from a magnification of ×54.

other recipients are alive and well 2.5 years later.

Mouse leukemias.—In experiments 10 and 11, the highly malignant lymphatic leukemias of Ak mice did not grow in the eyes of the guinea pigs and rabbits. In fact, the transplants resorbed more rapidly than did human tissues in these animals. In experiment 11 the mouse leukemia grew well in the eyes of all Ak and CF_1 mice. These data are summarized in Table 2. All of the Ak mice injected intraocularly died with leukemia between the twenty-second and thirty-ninth days; the growth in the eyes of 2 of these mice had completely regressed by the twenty-fifth day. Only one CF_1 mouse with intraocular transplant died with leukemia (twenty-third day). The other 9 intraocular transplants regressed and all were completely gone by 60 days. The first regression in these animals was seen at 25 days. Histologically, the eyes of the Ak mice contained leukemic cells in all chambers and infiltrating all orbital structures. There was little necrosis of the tumor tissue; it contained a few blood vessels. The eye of one CF_1 mouse that died was similar but had more necrosis in the tumor, no vascularization and many degenerating and atypical leukemic cells.

All Ak mice, injected subcutaneously, died with leukemia; the average survival time was 19 days. One CF_1 mouse survived, along with those injected intraocularly, for an average of 18 months. None developed leukemia or hematopoietic disease. Nine died with pulmonary tumors and the other with nephritis or pneumonia.

COMMENTS

These observations demonstrate that malignant lymphomas did not grow following transplantation into the ocular anterior chamber of alien species. The invasiveness and metastasizing ability of the diseases used in these experiments were high as evidenced by the short course of the diseases in the donors and their generalized distribution at physical or necropsy examination. Growth of lymphatic leukemias was obtained in eyes of homologous species but regression proceeded rapidly unless the tumors were in eyes of the homologous strain. In so far as one can generalize from this limited number of experiments, these observations would seem to set this group of neoplastic diseases apart from the others which have been shown to grow in heterologous eyes after they have developed metastases in the host. Present behavior can, however, be explained on other bases than nonmalignancy and/or absence of autonomy, which are the explanations given by Greene (10, 11, 12) for failures to grow.

According to Greene (12), (a) normal adult tissues, (b) inflammatory lesions, and (c) benign tumors fail to survive while (d) embryonic tissues and (e) malignant neoplasms grow after intraocular transplantation in heterologous species. If this view is correct, failure of these malignant lymphomas to survive after transplantation means that they belong in one of the first three categories. Yet, in an homologous strain these diseases, in the two tests made, not only survived but infiltrated widely locally and metastasized to all parts of the body, killing the animals. In their biological behavior they conformed to the accepted criteria of malignant neoplasms. The deductions which can be made from these observations are that either there exist non-neoplastic diseases whose manifestations are indistinguishable from those of neoplasms, or that successful intraocular transplantation in alien species is inadequate as a test for malignancy, it being too severe. The host may die from the effects of a vigorously growing mass of atypical cells from which there has been dissemination and formation of secondary masses in other parts of the body, yet, according to the view of Greene, the disease is not necessarily an autonomous malignant neoplasm. At this point the laboratory is deserted in favor of the field of semantics.

It is accepted that there are in tumors different degrees of malignancy. No doubt there are also different degrees within the definition of autonomy. To set up a standard (such as ability to grow after transplantation in the anterior ocular chamber of an alien species) and state that all tumors which do not meet this standard are not malignant and autonomous for the homologous individual, or even species, is to change the previously accepted definition for this word.

Failure of takes with these malignant lymphomas might, however, be explained on several other bases: (a) lack of resistance of these cells to various types of trauma and adverse conditions; (b) short life span of these cells; or (c) the rapid development of immunity.

Cells of the hematopoietic system are notoriously susceptible to injury. Temperature changes, radiations, adrenocorticotrophic and adrenocortical hormones, nonspecific heterologous body fluids, various drugs and metabolites all are capable, together with other causes, of injuring these cells under certain conditions. Transplants are put into an environment which is hostile. Some cell death is to be expected. But the cells of a malignant lymphoma, as contrasted with the hardier cells of fibrosarcomas and certain other neoplasms, would be expected to die at a high rate due to their susceptibility to noxious influences of these non-

specific types. It might be expected, therefore, that an insufficient number of cells might survive in a malignant lymphoma to make a take possible.

Successful transplantation obviously depends on survival of the cells. Length of survival is determined by the natural life span of the cell and on its ability to withstand unfavorable environmental conditions. No information exists on the life span of malignant lymphoma cells but it is probably no longer than that of the corresponding normal types of hemopoietic cells; these normally have a life span which is among the shortest for any type of tissue (3). If the cells die because of their short life span before a new source of nutrition is established, the transplant will resorb. However, successful growth of a malignant lymphoma of mice in homologous and heterologous strains shows that short life span alone did not prevent takes. The failures of human lymphomas to grow must therefore be explained with the aid of additional factors.

Failure of takes with these malignant lymphomas might be explained on the basis of a rapid development of immunity. This factor is seemingly dependent on the previous two factors. The cells normally have a life span which is among the shortest exhibited by any type of tissue. They are subjected to adverse environmental influences. After transplantation early necrolysis of some of the transplanted cells from the above mentioned two causes might release antigen which could quickly arouse a humoral defense reaction (9). According to Greene and others (1, 9) the anterior chamber is not isolated but it exhibits antibodies formed elsewhere. The entrance of these antibodies into the eye might augment the nonspecific causes for cell death. The successful transplantation of a mouse leukemia into recipients of the same species, under which conditions antibody formation and cell death would be at a minimum, shows that the immune reaction might be the determining factor in failure of heterologous transplants. Furthermore, total body x-radiation, which inhibits antibody production (2, 13), apparently enabled a canine lymphoma to take in an irradiated dog whereas the same tumor transplanted to a nonirradiated littermate failed to grow. At the same time the failure of these two lymphomas to grow in alien species indicated, therefore, not that they lack high malignancy and autonomy, but merely that immunological and other factors were probably operating.

If short life span and high susceptibility to cell injury resulting in early cell death provide antigen for antibody production, and if antibodies can unfavorably influence intraocular transplants, then the success or failure of intraocular transplantation may be dependent less on the inherent autonomy and malignancy of cells, than on the other three factors mentioned. This might explain the successful takes of brain tumors (of low grade malignancy but long cell survival) (10), of fibrosarcomas (5, 8), of chondrosarcomas (11), carcinomas of the prostate (4), and others. Likewise it could explain the failures in the highly malignant tumors of childhood (which probably consist of comparatively short-lived, labile cells) (5), and the malignant lymphomas.

This technic was of no aid in differential diagnosis in these malignant lymphomas. A review of the reports in the literature give little support to this test as a practical diagnostic procedure. The largest series of tests reported is that of Eichwald (5) who found 1 positive case in 27 malignant tumors in children (1 guinea pig positive in 158 tested). Greene (7) obtained 4 positive cases in 11 tested. Masina reported negative results with 3 carcinomas of the prostate (14), but of three kidney tumors 2 were positive (15). Many papers report positive results but fail to state how many clinically malignant tumors tested during the same period of time were negative. A tabulation or statistical analysis of the reports in the literature up to the present time is, therefore, not justified as it would be unfairly weighted in favor of the positive results. Even so, the reported negative cases outnumber those that were positive. This test as it now stands does not appear to offer great promise as a practical diagnostic procedure. Nevertheless the procedure has merit as offering a means for studying the biology of certain cancers.

SUMMARY

Intraocular transplantations in 7 cases of human malignant lymphomas (leukemia, lymphosarcoma, and Hodgkin's disease), 1 canine malignant lymphoma, and 2 leukemias of mice were unsuccessful in alien species (guinea pigs, rabbits, dogs, and mice). The mouse leukemias were transplantable in the eyes of homologous and heterologous strains of mice, but regression of the disease occurred rapidly in the latter strain. Histologic fate of transplants was rapid necrosis and partial resorption followed by organization of the debris.

The interpretation given to these experiments is that the failure of successful intraocular transplantation of malignant lymphomas in alien species was the result of the short life span of the cells, their high susceptibility to adverse conditions, and their antibody-engendering capacity, rather than to lack of a high degree of malignancy and autonomy.

This procedure was not of value in differential diagnosis.

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The Amino Acid Composition of a Fibrosarcoma and Its Normal Homologous Tissue in the Rat*

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About half of the amino acids which occur in proteins have been determined in cancer proteins by methods and investigators referred to by Toennies (2) in his excellent review (1947) entitled, "Protein-Chemical Aspects of Cancer." Both increased and decreased content of some amino acids in the proteins of malignant compared to homologous, normal tissues has been reported. Toennies has stated that although, ". . . the available evidence speaks against a striking gross difference between 'cancer protein' and 'normal protein' . . . ," this conclusion is based on evidence which is incomplete and may be faulty.

The view persists, nevertheless, that "the abnormal behavior of cancer cells is an expression of abnormalities in their chemical make-up." If this be true, it is still uncertain whether or not the proteins of cancer and homologous normal tissues have the same or different amino acid composition. It seemed worth while, therefore, to reinvestigate this problem with the aid of recently developed microbiological methods which have the advantage of high reproducibility, reasonable accuracy and ready applicability to nearly all of the commonly-occurring amino acids. In these studies it is recognized, as stated further by Toennies, that "identity of one protein fraction (or several) obtained from two kinds of tissue does not prove identity of the protein spectrum of the two tissues, nor does quantitative equality of one amino acid fraction (or several) obtained from two protein fractions prove identity of the amino acid spectrum of the two proteins."

Twelve amino acids (L-forms) of a fibrosarcoma and its normal homologous tissue in the rat have been determined in the present investigation.

EXPERIMENTAL

Male and female rats of the Long-Evans strain were maintained on the Anderson-Smith (3) and the Rockland-pellet diets with supplements of a weekly ration of lettuce and unlimited water. Active fibrosarcoma transplants (series no. 8), originally induced by subcutaneous injections of methylcholanthrene (4) and carried through numerous generations of Long-Evans rats (Z, Table 1), were obtained directly from the Zoology Department and were injected subcutaneously into Long-Evans carrier rats (A and P, Table 1). In making transplants the active tumor growth was dissected aseptically from carrier rats under nembutal and ether anaesthesia. The excised tumor material (1 part) was immersed in sterile physiological saline (4 parts) and ground to a fine suspension in a sterile Waring blender.

The rats were inoculated as follows: The animals were anaesthetized and the skin areas were shaved and sterilized. *Subcutaneous inoculation:* A skin incision (1.0 mm. in length) was made along the midventral body line at the level of the lower border of the kidney. A 1.0 ml. volume of the tumor suspension was injected through the skin opening into each of four subcutaneous (bilateral axillary and inguinal) sites with a 30 ml. syringe and a no. 15 hypodermic needle. Backward trailing of the tumor suspension as the needle was withdrawn was prevented by manual pressure at the delivery site. *Intramuscular and intraperitoneal inoculation:* Injections were made similarly into the proximal third of the striated muscle of all four limbs and the lower peritoneal cavity, respectively. *Intrarenal inoculation:* The left kidney was delivered from the peritoneal cavity of the anaesthetized animal through a transverse suprarenal incision in the lateral body wall. Approximately 0.1 ml. of the tumor suspension was injected through the renal capsule into the upper pole of the kidney parenchyma. Hemostasis was obtained with gelfoam-thrombin packs and the capsule was closed with 5-0 chromic suture in an eye-atraumatic needle. The muscle was approximated with 5-0 chromic suture and the skin was closed with clips.

Representative samples of all tumor and normal tissues analyzed were prepared for microscopic

* Paper 58. For Paper 57, see Dunn *et al* (1). This work was aided by grants from the American Cancer Society through the Committee on Growth, National Research Council, and from the Cancer Institute, University of California. The authors are indebted to Dr. M. N. Camien for helpful suggestions.

study. Sections were 5 to 6 micra thick and were stained with (Harris) hematoxylin and eosin.

The normal and malignant tissues were prepared for microbiological assay as follows: The tissue was extirpated from the anaesthetized animal, dissected free from extraneous material (including the connective tissue capsule of the tumor), weighed immediately and transferred to a bottle containing a quantity of 8 *N* HCl five times the weight of the moist tissue. The bottle was stoppered and stored in the refrigerator until the acid mixture was hydrolyzed by refluxing it for 24 hours at 130° C. The acid hydrolysate was filtered, bottled and stored in the refrigerator until it was assayed for 12 amino acids by the microbiological procedures of Dunn *et al* (5).

Moisture, fat (ether-soluble material), collagen and elastin were determined by the methods of Lowry *et al* (6). Ash was determined by heating the fresh and the moisture- and fat-free tissues to constant weight in a muffle furnace.

RESULTS

The initial and final weights of the rats, the site of the transplants, the ages and weights of the tumors and other data for the normal and the experimental animals are given in Tables 1 and 2.

The subcutaneous tumor mass was firm, lobu-

TABLE 1

DATA ON RATS AND RAT FIBROSARCOMAS

	Rat			Tumor	
Description*	Age (months), time of transplant	Weight (grams), time of transplant	Weight (grams), terminal‖	Weight (grams)	Age (days), transplant to extirpation
		(Subcutaneous)			
A	3.5	275	244	37.3	15
A†	5.0	410	397	46.6	18
A†	5.0	238	205	33.9	19
Z	7.0	279	290	55.0	21
P†	3.0	406	363	58.8	16
P†	3.0	452	365	82.0	14
P†	3.0	445	276	82.1	18
P†	3.5	460	385	67.0	17
Z¶	10.0	230	178	65.5	16
Z¶	10.0	180	91	36.4	16
Z¶	10.0	228	102	41.6	16
Z¶	10.0	216	193	25.7	19
Z¶	10.0	200	199	18.4	20
Z¶	10.0	222	238	16.9	20
Z¶	10.0	200	204	20.0	20
		(Intramuscular)			
A‡	5.0	410	379	62.1	16
A‡	5.0	447	419	49.7	15
A‡	5.0	350	283	42.0	12
		(Intrarenal)			
A	10.0	310	268	5.8	22
A	11.0	343	326	51.1	29
A	11.0	337	323	4.9	16
A	11.0	384	377	12.6	16
A	11.0	336	321	2.9	17
		(Intraperitoneal)			
A§	10.0	378	337	12.2	16
A§	10.0	380	362	30.2	21
A	10.0	390	399	35.0	19
A	10.0	420	458	31.3	51
A	10.0	262	262	10.7	11
A	10.0	313	280	29.7	15
A	10.0	245	243	18.9	10

A, Authors' (Long-Evans) colony initiated in 1943 from rats obtained from the Philip R. Park Foundation, San Pedro, California.

P, Philip R. Park Foundation (Long-Evans) colony initiated in 1927 from rats obtained from Dr. H. R. Evans, University of California, Berkeley, Calif.

Z, U.C.L.A. Zoology (Long-Evans) colony initiated in 1928 from rats obtained from Dr. H. R. Evans.

* Except as noted all of the rats were hooded males maintained on Rockland pellets.

† Rockland pellets and Anderson-Smith diets.

‡ Anderson-Smith diet.

§ Albino.

‖ Exclusive of tumor weight.

¶ Female.

lated and encapsulated with a thick connective tissue wall. Occasionally the underlying muscle was invaded but metastasis to distant sites was not encountered. The cut surface presented well-vascularized, grayish-white tissue. Slight modifications were noted in the anatomy of tumors implanted in the other sites. For example, the capsule was often thin or obliterated by the invasive tumorous tissue, vascularization was increased and metastasis by direct extension or by way of the blood stream was observed.

Histological examination of tumor tissue sections collected from the four sites revealed a typical fibrosarcoma characterized cytologically by a predominance of large spindle cells with vesicular nuclei which exhibited, commonly, enlarged nucleoli and different stages of mitosis. Giant cells with very large round nuclei and frequent mitotic figures were seen occasionally among the more prevalent fusiform cells. Supporting interstitial tissue was scant. Collagenous fibers and fibrils separated the fibroblastic tumor cells into bundles which were parallel or at angles to each other and which often formed criss-cross patterns or whorls in the limited stroma. Thin-walled blood vessels, capillaries and sinusoids appeared as a vascular framework for the rapidly proliferating tumor cells. Necrotic areas were present in a few sections. In contrast, the classical histological anatomy of the normal adult connective tissue was characterized by a dearth of nuclear material, the fibroblast being the typical cell. Abundant collagenous fibers and a few elastic fibers appeared in its stroma. The marked increase in cellularity and the dearth of stroma in the malignant compared to the normal tissue were clearly evident.

TABLE 2

DATA ON RATS AND RAT NORMAL SUBCUTANEOUS CONNECTIVE TISSUE*

Description	RAT Age (months), terminal	Weight (grams), terminal	TISSUE WEIGHT (GRAMS)
A†	5.0	344	8.4
A†	4.5	325	10.2
A†	5.0	334	13.5
A†	5.0	345	11.0
P‡	4.0	420	21.6
P	4.0	306	20.5
P†	4.0	313	25.4
P†	4.0	256	26.4
Z¶	7.0	213	6.5
Z¶	6.0	223	7.3
Z¶	8.0	238	8.3
Z¶	7.0	233	7.1
Z¶	7.0	201	5.6
Z¶	8.0	201	5.9

* See footnotes to Table 1 for explanation of symbols.

Data on the amino acid composition of the fibrosarcoma and its homologous normal connective tissue are given in Tables 3 to 7. Since the amino acid values were closely agreeing for different samples of the normal as well as of the malignant tissues (Table 3), the pooling of samples from each type of tissue appeared to be justified. The values found, subsequently, for the pooled samples differed from the averages of the individual samples by less than 10 (0 to 28) per cent. Similarly, the sarcomas obtained by inoculation at the four sites had nearly the same amino acid composition calculated either on the wet weight basis (Table 4) or as the per cent of the total amino acids determined (data not shown). On the other hand, the content of some amino acids (arginine, glycine, isoleucine, leucine, lysine, threonine and valine) in normal connective tissue appeared to differ significantly from that in malignant tissue (Table 4).

It is of particular interest that the dry, fat- and ash-free connective tissue contained 65 per cent collagen compared to only 3 per cent for the corresponding malignant tissue (see footnotes to Table 5). The collagen value for normal tissues is approximately the same as that found by Lightfoot and Coolidge (43) in guinea pig fascia.

The amino acid composition of the normal and malignant tissues (moisture-, fat-, and ash-free basis), corrected for the amino acids in collagen and elastin on the basis of the data given in Table 6, is given in Table 5. The amino acid values represent the amino acid composition of the cellular proteins *per se* if it is assumed that the nitrogen originated almost wholly from proteins. Accepting this premise, the arginine and glycine content of the fibrosarcomatous proteins was markedly higher than that of the normal connective tissues. It is evident, also, that threonine was increased and that there were significant decreases in some amino acids, particularly histidine and methionine. Comparable differences between the amino acid values were observed for the female rats (Table 7). It was concluded from these results that there were no significant variations in amino acids due to the age, sex, diet, or the colony of rats employed.

DISCUSSION

Numerous data have been reported in the literature on some of the amino acids in albumins, globulins, nucleoproteins, phosphoproteins, and other kinds of protein fractions derived from various types of neoplasms. It seems probable that many of these data are inaccurate because of the unreliability of the methods employed (such as determination of the basic amino acids by the

silver-salt and the nitrogen-distribution procedures, glycine and glutamic acid by isolation, and cystine and tryptophan by colorimetry).

Previous investigators have concluded that certain amino acids were increased, decreased or unchanged in tumor tissue compared to the homologous normal tissue. An increase in the diamino acids (7 to 13) and in arginine (10, 11, 15 to 18), tyrosine (19), and methionine (14, 20), and a decrease in tryptophan (21 to 25) and cystine (14, 20, 26) have been reported. Other data have been conflicting or inconclusive for lysine and histidine (10, 11, 15 to 17), tryptophan (18, 22, 23, 25, 27 to 29), tyrosine (18, 24, 30), glutamic acid (30) and glycine (30).

Whether or not such differences occur in the cellular proteins *per se* or are artifactitious as has been suggested by Drummond (11), is a critical problem. It would be possible, theoretically, to isolate the "pure" proteins and to determine their amino acid composition but it would be difficult, if at all possible at the present time, to fractionate quantitatively the intracellular and the extracellular proteins. On the other hand, Lowry and Hastings (44) have developed methods for the determination of the extra- and intracellular portions of various tissues. These methods have been applied in studies on a number of problems (45 to 51). The corrections made in the present study for moisture, fat, ash, collagen and elastin, represent application of the pertinent parts of these methods. It was deemed unnecessary to correct for the amino acids in the blood and interstitial fluids of the tissues investigated since it can be shown, on the basis of

TABLE 3

PERCENTAGES OF AMINO ACIDS IN (MALE) RAT NORMAL CONNECTIVE TISSUE AND SUBCUTANEOUS FIBROSARCOMAS

	Normal tissue						Tumor tissue						Over-all average	
	Individual rats				Average	Pooled samples†	Individual rats				Average	Pooled samples†	Tumor tissue (8 rats)	Normal tissue (8 rats)
Amino acid*														
Arginine	0.56	0.53	0.57	0.47	0.53	0.58	0.69	0.63	0.64	0.75	0.68	0.69	0.68	0.56
Aspartic acid	0.62	0.61	0.60	0.68	0.63	0.69	1.03	0.75	0.80	1.06	0.91	1.05	0.98	0.66
Glutamic acid	0.89	0.90	0.88	0.96	0.91	0.98	1.30	1.28	1.31	1.58	1.37	1.41	1.39	0.94
Glycine	1.16	1.21	1.20	1.16	1.18	1.48	0.53	0.56	0.56	0.49	0.53	0.65	0.59	1.33
Histidine	0.17	0.15	0.16	0.14	0.16	0.15	0.25	0.22	0.22	0.27	0.24	0.27	0.26	0.16
Isoleucine	0.23	0.23	0.23	0.27	0.24	0.25	0.50	0.49	0.50	0.54	0.51	0.50	0.50	0.24
Leucine	0.43	0.45	0.45	0.46	0.45	0.43	0.85	0.83	0.79	0.95	0.86	0.91	0.89	0.44
Lysine	0.46	0.47	0.49	0.48	0.47	0.49	0.84	0.79	0.80	0.97	0.85	0.86	0.86	0.48
Methionine	0.13	0.20	0.16	0.09	0.14	0.10	0.24	0.24	0.24	0.28	0.25	0.21	0.23	0.12
Phenylalanine	0.27	0.25	0.25	0.25	0.25	0.25	0.39	0.37			0.38	0.47	0.43	0.25
Threonine	0.28	0.30	0.25	0.22	0.26	0.19	0.50	0.53	0.52	0.55	0.52	0.43	0.48	0.22
Valine	0.34	0.34	0.36	0.35	0.35	0.34	0.58	0.52	0.55	0.63	0.57	0.60	0.58	0.34
Nitrogen*	1.34	1.31	1.37	1.36	1.34	1.38	2.29	1.60	1.71	1.98	1.90	1.73	1.82	1.36

* Per cent of wet weight of tissue.
† Four rats.

TABLE 4

PERCENTAGES OF AMINO ACIDS IN (MALE) RAT FIBROSARCOMAS FROM DIFFERENT SITES

	Fibrosarcoma*				Fibrosarcoma Average, all sites (23 rats)			Normal connective tissue (8 rats)‡	
Amino acid	Subcutaneous† (8 rats)	Intramuscular (3 rats)	Intraperitoneal (7 rats)	Intrarenal (5 rats)	Wet wt.	Dry, fat- and ash-free wt.§	16% N basis	Dry, fat- and ash-free wt.	16% N basis
Arginine	0.68	0.75	0.67	0.74	0.71	5.1	6.0	6.7	6.6
Aspartic acid	0.98	1.04	0.96	0.97	0.99	7.2	8.4	7.9	7.7
Glutamic acid	1.39	1.52	1.29	1.35	1.39	10.1	11.9	11.3	11.0
Glycine	0.59	0.73	0.59	0.70	0.65	4.7	5.5	16.0	15.7
Histidine	0.26	0.29	0.29	0.11	0.28	2.0	2.4	1.9	1.9
Isoleucine	0.50	0.51	0.47	0.55	0.51	3.7	4.4	2.9	2.9
Leucine	0.89	0.86	0.82	0.90	0.87	6.3	7.4	5.3	5.2
Lysine	0.86	0.86	0.84	0.93	0.87	6.3	7.4	5.8	5.6
Methionine	0.23	0.18	0.18	0.16	0.19	1.4	1.7	1.4	1.4
Phenylalanine	0.43	0.43	0.44	0.48	0.44	3.2	3.7	3.0	3.0
Threonine	0.48	0.41	0.39	0.44	0.43	3.1	3.7	2.7	2.5
Valine	0.58	0.60	0.58	0.65	0.60	4.3	5.1	4.1	4.0
Nitrogen	1.82	1.86	1.87	1.96	1.88	13.6	16.0	16.3	16.0

* Wet-weight basis.
† Data from Table 3.
‡ Values calculated from data in Table 3.
§ Values lower than 16 per cent nitrogen basis probably because of non-protein substances including phospholipids (55 to 62) and carbohydrates (14, 63 and 64).

data in the literature (5, 42, 44, 48), that even in a tissue so vascular as liver, the corrections representing the proteins of the blood and interstitial fluid fall well within the probable experimental error of the microbiological values for the amino acids.

TABLE 5

PERCENTAGES OF AMINO ACIDS IN PROTEINS* OF (MALE) RAT NORMAL CONNECTIVE TISSUE AND A FIBROSARCOMA

Amino acid	Normal connective tissue†	Fibrosarcoma‡
Arginine	2.8	5.9
Aspartic acid	10	8.6
Glutamic acid	11	12
Glycine	0§	4.5
Histidine	4.0	2.5
Isoleucine	4.8	4.5
Leucine	8.5	7.5
Lysine	7.6	7.5
Methionine	2.5	1.8
Phenylalanine	4.0	3.8
Threonine	2.8	3.8
Valine	4.8	5.2

* Assumed to be same as N × 6.25. The tissues were corrected for the amino acids in collagen and elastin on the basis of the values given in Table 6. It is of interest that, the amino acid values for gelatin are in reasonably good agreement with those reported recently by Bowes and Kenten (41) and Graham *et al.* (65) for collagen.

† Composition of moist tissue was as follows: Nitrogen, 1.36 per cent; moisture and fat, 91 per cent; ash, 0.66 per cent; collagen, 5.4 per cent; elastin, 0.06 per cent; and protein, 8.5 per cent (N × 6.25) and 8.3 per cent (collagen-, elastin-, moisture-, ash- and fat-free residue).

‡ Composition of moist tissue was as follows: Nitrogen, 1.88 per cent; moisture and fat, 85 per cent; ash, 1.2 per cent; collagen, 0.47 per cent; elastin, 0.05 per cent; and protein, 11.8 per cent (N × 6.25) and 13.9 per cent (collagen-, elastin-, moisture-, ash-, and fat-free residue).

§ Within experimental error.

The present results are in agreement with those of earlier workers who found that arginine was increased in malignant tissues. Even though the ratio of nucleated to fibrous material is higher in neoplastic than in normal connective tissue, it seems probable that the authors' corrected arginine values represent nearly the true percentages of this amino acid in the cellular proteins of these tissues. It is of interest in this connection that, according to Wilkerson and Gortner (32), the arginine content of embryonic tissue is inversely proportional to the age of the embryo, and of neoplasms, to the degree of malignancy. On the other hand, Bach and Lasnitzki (33) have shown that arginine and ornithine accelerated the *in vitro* mitosis of mouse adenocarcinoma 63, but not of normal embryonic mouse lung, and that slow-growing tissue contained twice as much arginase as fast-growing tissue of the same tumor strain. The importance of arginine in embryonic as well as autonomous growth has been emphasized by Irons (31), and it may be that the high proportion of arginine in neoplasms indicates some critical function of this amino acid in malignant growth. It has been reported by Beard (34) and other workers (35 to 36), that transplanted tumors in rats regressed following the administration of arginine and some other amino acids. A similar effect of amino acids was assumed by Waterman *et al* (36) who found that the increase in oxygen uptake by tumor tissue was less than that of normal tissue when both types of tissue were tested in the presence of spleen extract.

That there may be some type or degree of derangement in the metabolism of creatine and nucleoproteins is indicated by the striking increase of glycine and the decrease of histidine and methionine in tumor tissue observed in the present studies. Greenstein (37) has found, however, that there was no difference in the creatine content of

TABLE 6

AMINO ACID COMPOSITION OF GELATIN, COLLAGEN, AND ELASTIN

	Gelatin			Collagen		Elastin	
Amino acid	Authors' data*	Block & Mitchell†	Graham *et al.*‡	(Achilles tendon) Graham *et al.*‡	(Isolated collagen) Bowes & Kenten§	(Isolated elastin) Graham *et al.*‡	(Tendon) Block & Mitchell†
Arginine	9.0	8.7	7.3	7.6	8.8	1.1	1
Aspartic acid	6.3		6.1	6.5	6.3	0.6	
Glutamic acid	11.4		9.9	10.5	11.3	3.2	
Glycine	26.4	24–30			26		27
Histidine	0.74	0.9	0.72	0.85	0.8	0.04	0
Isoleucine	1.5	1.7	1.3	1.8	5.6‖	3.3	
Leucine	3.3	3.1	3.2	3.3		7.0	
Lysine	4.4	5.8	3.7	3.3	4.5	0.05	?
Methionine	0.91	0.8	0.91	0.85	0.8	0.03	0.4
Phenylalanine	2.2	2.1	2.0	2.4	4.2	4.6	3.1
Threonine	2.1	2.0	1.7	2.4	2.4	1.1	2.5
Valine	2.8	2.8	2.4	2.7	3.4	13.0	

* Authors' data (unpublished) for purified gelatin calculated to 16 per cent nitrogen.

† Block and Mitchell (42). Data calculated to 16 per cent nitrogen.

‡ Graham *et al.* (65). Data calculated to 16 per cent nitrogen.

§ Bowes and Kenten (41). Data calculated for tissue on water-, ash-, and fat-free basis.

‖ Sum of isoleucine and leucine.

normal, regenerating and cancerous liver of the mouse or rat. On the other hand, Greenstein and Leuthardt (20, 26) have shown that the relatively high methionine and low cystine content of hepatomas indicates that the latter are related more closely to fetal liver than to the normal or regenerating liver of adults. It may be of further significance that cystine increased while total sulfur remained constant in the developing frog embryo (38), that lysine increased while arginine and histidine decreased in the developing pig embryo (32), and that the glycine-containing tripeptide, glutathione, increased in a transplanted hepatoma (2).

TABLE 7

PERCENTAGES OF AMINO ACIDS IN (FEMALE) RAT NORMAL CONNECTIVE TISSUE AND A FIBROSARCOMA

Amino acid*	Normal subcutaneous tissue (6 rats)	Subcutaneous fibrosarcoma (7 rats)
Arginine	0.34	0.76
Aspartic acid	0.49	1.08
Glutamic acid	0.67	1.45
Glycine	0.82	0.65
Histidine	0.27	0.23
Isoleucine	0.19	0.51
Leucine	0.33	0.83
Lysine	0.36	0.77
Phenylalanine	0.17	0.47
Threonine	0.21	0.46
Valine	0.25	0.56
Nitrogen	0.92	1.74

* Moist-tissue basis.

Christensen and his co-workers (39, 49 to 51) have applied the methods of Lowry *et al* (44) and others (53, 54) to the study of the free amino acids and peptides in fetal, regenerating and mature tissues. They have shown that, in general, the increase in the concentrations of free amino acids (particularly glycine) and glutathione in the plasma and in the cells may be correlated with increase in mitotic rate and that the intracellular increase in concentration is relatively greater than the extracellular. These results agree with the more recent findings of Roberts and Tishkoff (52), using chromatographic methods, that "The epidermis of newborn mice and hyperplastic epidermis, both of which tissues have a more rapid rate of growth than normal adult epidermis, had greater concentrations of free amino acids than the latter tissue, ..." They also found, however, that a "rapidly-growing carcinoma had a much lower content (of free amino acids) than normal" and concluded that, "This suggests that the growth in the non-malignant epidermis may be associated with the ability of the cells to increase the intracellular concentrations of the amino acids necessary for protein synthesis, whereas in the malignant tumor the mechanisms for protein synthesis are much more efficient and can operate at a greatly accelerated rate, even in the presence of smaller concentrations of amino acids." These authors reported further (unpublished data) that "it was found that the amino acid pattern of whole tumor tissue was significantly different from that of normal epidermis, but similar in most respects to that of the nonmalignant hyperplastic epidermis produced by the application of methylcholanthrene" but that "the carcinoma can be sharply differentiated from the latter tissue on the basis of the distribution of free amino acids in the alcoholic extracts."

SUMMARY

The percentages of 12 amino acids in rat fibrosarcoma and its normal homologous tissue have been determined by microbiological methods. The most significant differences found were a markedly higher content of arginine and glycine, an increase in threonine, and a lower content of histidine and methionine in the malignant than in the normal tissue. Since the amino acids were calculated for the tissues corrected for moisture, ash, fat, collagen and elastin, they were assumed to be approximately the values for the cellular proteins. No significant differences in the amino acid composition were observed for fibrosarcomas grown in four different sites in male and female (Long-Evans) rats obtained originally from the same source but maintained for many years on different diets in separate colonies. The possible physiological significance of these experimental results has been discussed.

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Glycolytic Enzyme Inhibitor Therapy in Human Malignant Neoplasia*

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The pioneer studies of Warburg on the metabolism of tumor tissue have been corroborated and extended by an increasing number of investigators. In fact, criteria of malignant metabolism have been established, enzymatic patterns of malignant tumors defined, and the uniformity of malignant neoplasia indicated by the observation that tumors tend to converge enzymatically to a common type of tissue (3) (4).

The accentuated aerobic and anaerobic glycolysis of cancer tissue formed the basis for an attempt to selectively inhibit tumor energetics in a previous study (1). It was suggested that with the advent of malignancy there was a significant alteration in the energy mechanism of tissues. The preferred pathways would involve the glycolytic mechanism while the respiratory enzyme reactions were diminished as compared to their role in normal tissue. However, the respiratory functional potentiality would not be lost but would remain residual. On this hypothesis, various inhibitors of glycolysis as well as sodium azide were used in the treatment of acute leukemia, lymphomas, and a limited number of other cancer types. It is the purpose of this paper to extend this study by a report of the effects of fluoride, iodoacetate, and malonate on a series of more than one hundred cases of diverse forms of human malignant neoplasia, with particular reference to the question of basic uniformity of cancer tissue metabolism.

The clinical effects of treatment of neoplastic disease depend not only on the particular therapeutic agent and the cancer type but also on such factors as the general status of the patient at the time of treatment, and the incidence of pre-treatment. Thus, radiation therapy, while effective initially in the treatment of lymphomas, gradually fails to influence the growth after repeated occurrence. It is not the purpose of this paper to decide on the relative clinical efficacy of these compounds but rather to note if possible any differential sensitivity of various cancer types and evaluate the observations in terms of the theoretical mechanisms of action in relation to *in vitro* studies of the metabolism of cancer tissue.

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MATERIALS AND METHODS

The cancer[1] cases studied were for the most part far advanced and beyond the aid of surgery or radiation therapy, and in almost all cases the diagnosis was confirmed by biopsy. The enzyme inhibitors studied included sodium fluoride, iodoacetic acid, malonic acid, and sodium azide. For the most part these agents were utilized in the manner and dosage indicated in our previous paper; that is, the glycolytic inhibitors fluoride and iodoacetate were considered as a primary group, with the malonate used as an adjunct to them, while the azide was employed separately. These drugs were administered orally in the following average adult doses: sodium fluoride,[2] 80 mg. q.i.d., iodoacetic acid, 30 mg. b.i.d., malonic acid, 0.5 gm. b.i.d., and sodium azide, 0.7 mg. t.i.d. In addition intravenous administration of the glycolytic inhibitors was employed in a smaller series of cases. This latter procedure was used to see if any significant differences would be obtained when the route of administration varied, and also to remove any uncertainty of absorption or alteration in the gastrointestinal tract.

The effects of administration of these agents were divided into subjective and objective manifestations, and the extent of any response classified as good, fair, and poor. This procedure allows a rapid survey of the degree and type of effects obtained with the treatment.

RESULTS

The majority of the patients with varied tumor types failed to experience either subjective or objective beneficial effects from these agents when

[1] The term cancer as used here refers to all types of malignant neoplasia, carcinoma and sarcoma.

[2] Kindly supplied by Endo Products, Inc., Richmond Hill, N.Y.

employed in the manner indicated. However, this was not the case in certain of the groups where significant objective and subjective benefits were observed. On the basis of the cases studied, we have divided the various cancer types into three categories: (1) Responsive, (2) Non-Responsive, (3) Questionably Responsive. Again it should be emphasized that this classification and the ensuing data refer predominantly to the observation of a "cause and effect" relationship between the administration of the fluoride, iodoacetate, and malonate, and alterations in subjective and objective findings of the patient. A more detailed report will be made at a later date covering the use of these agents in the Responsive group.

Summation of the response obtained in the three groups may be found in Tables 1, 2, and 3. The criteria and constitution of the groups are as follows: 1) Responsive—definite and reproducible evidence of regression of the malignant growth, accompanied by symptomatic improvement within a period of one month's treatment. This group includes Hodgkins disease, lymphosarcoma, and acute leukemia. 2) Non-Responsive—no evidence of regression of malignant growth or symptomatic improvement after one month's treatment. Included here are colon carcinoma, uterine fundus carcinoma, chronic leukemia, melanoma, ovarian carcinoma, pancreas carcinoma, rectal carcinoma, squamous cell carcinoma of the cheek and pharynx, and testis choreonepithelioma. 3) Questionably responsive—suggestive but not conclusive evidence of beneficial effects of treatment, *viz.*, transient symptomatic improvement without real regression of the tumor or apparent enhancement of beneficial effects of other modalities of therapy *e.g.* radiation, or favorable effects but inadequate data for definite opinion. In this group are breast carcinoma, carcinoid, cervix carcinoma, lung carcinoma, stomach carcinoma, and testis carcinoma.

More adequate classification of the latter group must await greater accumulation of data. The significant point at this time is the observation that marked differences exist in the sensitivity of various malignant neoplastic growths to the agents under discussion. In addition it is also noteworthy that within an individual responsive group variations occur. Thus, extensive prolonged pre-treatment of Hodgkin's disease by radiation seems to be associated with decreased response to the chemotherapeutic agents and more particularly to the glycolytic inhibitors. Such cases are sometimes sensitive to azide, which may in addition bring about a responsiveness to fluoride, iodoacetate, and malonate. This phenomenon was the subject of a previous report (2). However, there is no evidence that the use of any of these agents significantly prolongs life even in responsive cases, although they may increase useful and "healthy" life within the life span.

In acute myeloblastic leukemia, correlation appears to exist between the morphology of the malignant cell and the sensitivity to the chemotherapeutic agent. (1) The usual myeloblast is a relatively large cell with abundant basophilic cytoplasm and a vesicular nucleus containing well-defined nucleoli (Bl_o). Leukemia patients with a predominance of this type of cell usually experience a remission when treated with the glycolytic inhibitors. However, an insensitivity eventuates and is usually associated with a change in the morphology of the blast cell. The cytoplasm becomes markedly reduced, sometimes becoming almost non-existent, while the nuclear chromatin is condensed and the nucleoli tend to disappear. This cell, which we have called an adaptation blast (Bl_a), varies in size from 8 μ to 20 μ. When these cells predominate, glycolytic inhibitor therapy is without any appreciable effect. It should also be mentioned that this type of cell is also seen spontaneously in leukemia cases, or may occur following urethane treatment.

Examination of Tables 1 and 2 reveals a clear-cut difference in response to treatment in the disease forms of the two groups. It should also be mentioned that in some of the cases of Hodgkin's disease and acute leukemia, several successive remissions were induced by this form of treatment. Thus, in one case of Hodgkin's disease, eight instances of remission occurred coincident to treatment at different times over a 2-year period.

In the Questionably Responsive group some subjective and objective benefit appeared to be associated with chemotherapy in a significant number of cases. However, these changes were not as clear-cut and lasting as in the Responsive group.

TOXICITY

Oral administration of the glycolytic inhibitors was associated with nausea and diarrhea in approximately 25 per cent of the cases studied. However, the severity of these complaints varied markedly from slight gastrointestinal upsets of short duration to an inability to tolerate treatment. The latter type of response was more frequent in cases with carcinoma of the colon, particularly where extensive liver metastases were present. On the other hand, intravenous administration of considerably higher doses was not found to give rise to any significant systemic complaints. Some tendency toward the appearance of a chemical thromobophlebitis was encountered at the side of

TABLE 1

RESPONSIVE GROUP

DISEASE	No. CASES	RESPONSE Subjective* Good	Fair	Poor	Objective Good	Fair	Poor	SIGNS†
Hodgkin's	13	7	2	4	7	3	3	Decrease in adenopathy, hepatosplenomegaly, hyperpyrexia.
Lymphosarcoma	8	2	2	4	3	1	4	Decrease in adenopathy, hepatosplenomegaly, hyperpyrexia.
Leukemia, acute myeloblastic (Bl_0)‡	7	3	1	3	4	1	2	Decrease in adenopathy, hepatosplenomegaly, hyperpyrexia; disappearance of blast cells.
Leukemia, acute myeloblastic (Bl_a)	4	0	1	3	0	2	2	
Leukemia, acute lymphoblastic	4	3	0	1	3	1	0	Decrease in adenopathy, hepatospenomegaly, hyperpyrexia; disappearance of blast cells.
Total	36	15	6	15	17	8	11	

* Subjective benefits included gain in strength and appetite, and decreased pain.

† Changes observed coincident with glycolytic inhibitor therapy.

‡ Bl_0 refers to those cases in which the predominant myeloblast possessed abundant cytoplasm and well defined nucleoli. Bl_a refers to those cases in which the myeloblast had scanty cytoplasm and indistinct nucleoli.

TABLE 2

NON-RESPONSIVE GROUP

CANCER TYPE	No. CASES	RESPONSE Subjective Good	Fair	Poor	Objective Good	Fair	Poor	SIGNS
Colon	12			12			12	
Fundus uteri	2			2			2	
Leukemia, chronic	4			4			4	
Melanoma	4			4			4	
Ovary	3			3			3	
Pancreas	1			1			1	
Rectum	5			5			5	
Squamous cell, face, pharynx	2			2			2	
Chorionepithelioma testis	2			2			2	
Total	35	0	0	35	0	0	35	

TABLE 3

QUESTIONABLY RESPONSIVE GROUP

CANCER TYPE	No. CASES	RESPONSE Subjective* Good	Fair	Poor	Objective Good	Fair	Poor	SIGNS†
Breast	10		6	4		5	5	Regression of some skin lesions; gain in weight; decreased pleural effusion.
Carcinoid, colon	2		2			2		Biopsy evidence of unusual necrosis; gain in weight.
Cervix	1		1			1		Cessation of discharge; decreased leg edema.
Lung	8	1	4	3		4	4	Gain in weight; decreased pleural effusion.
Stomach	12		6	6		3	9	Gain in weight; increased ability to swallow food.
Testis	2		2			2		Decreased gynecomastia; decreased 17-ketosteroid excretion; relief of pressure symptoms.
Total	35	1	21	13	0	17	18	

* Changes observed coincident to treatment with glycolytic inhibitor therapy.

† Subjective benefits included gain in strength, gain in appetite, and decrease in pain.

the injection when the dose was concentrated. However, this could be overcome by diluting the medication in 250 cc. of saline, then giving it as an infusion. The following brief protocols indicate the doses used intravenously and provide examples of the three types of response groups:

1. *Responsive*—R. C., 42-year old white female, was first seen on May 27, 1948. About 1 month previously she had noted the appearance of an enlarged lymph gland in the left lateral aspect of the neck. It became somewhat tender and several other glands appeared in the same area. A slight diminution in size occurred spontaneously, although several new areas of involvement appeared. The patient also complained of gastrointestinal distress which was attributed to an ulcer which had been demonstrated on x-ray examination. Physical examination was essentially negative except for the presence of an enlarged chain of glands along the left lateral aspect of the neck. Blood count at this time was within normal limits. Biopsy revealed almost complete loss of follicular architecture, reticular cell hyperplasia, and scattered eosinophilis. These findings were considered suspicious but not diagnostic of Hodgkin's disease by 3 pathologists.

Although the glands appeared to undergo some partial diminution in size after the biopsy, inguinal adenopathy was noted on July 7, 1948, followed by axillary and increased cervical adenopathy. The patient received radiation therapy to the cervical and inguinal regions, but no appreciable regression occurred. Glycolytic inhibitor therapy was started on November 8, 1948, consisting of oral administration of sodium fluoride, 160 mg. t.i.d., iodoacetic acid, 43 mg. b.i.d., and malonic acid, 715 mg. b.i.d. Within a period of several weeks, the glands were seen to undergo definite regression in all areas, although definite residual adenopathy was present on December 16, 1948, at which time it was decided to employ intravenous administration. The patient received 5 injections over a period of 10 days, consisting of sodium fluoride, 130 mg., iodoacetic acid, 50 mg., and malonic acid, 1500 mg., all neutralized to pH 6.8. Following this there was a rapid regression of the glands. On January 15, 1949, the lymphadenopathy was still markedly reduced, although the inguinal chain appeared moderately prominent. The patient received only one x-ray treatment to this area and regression was noted promptly. At present (March 1949) adenopathy is minimal, and aside from the complaints attributable to the ulcer, the patient is in good health.

2. *Non-Responsive*—S. L., a 45-year old white female with an inoperable carcinoma of the rectosigmoid area and a functioning colostomy which had been established in July 1948. The patient complained of severe abdominal pain, anorexia, and weight loss. Glycolytic inhibitor therapy was started on October 29, 1948. From that date to November 11, 1948, the patient received sodium fluoride, 160 mg. t.i.d., p.o., and an average daily intravenous dose of iodoacetic acid, 57.3 mg./day, and malonic acid, 748 mg./day. From November 11 to November 30, the patient also received a daily intravenous administration of sodium fluoride, 293 mg./day.

Despite the prolonged administration of relatively high doses of the drugs, no beneficial effects were noted, and the patient expired in the latter part of December 1948, from the progression of the disease. Permission for autopsy could not be obtained.

3. *Questionably Responsive*—E. L., a 35-year old white female, noted a mass in the left breast in October 1945. The lesion proved to be a duct cell carcinoma, and a radical mastectomy was performed. The patient remained symptom free until March 1947, at which time metastatic bone defects were demonstrable in the left ilium, and in lumbar vertebrae 2 and 3. In addition, masses of tumor tissue were noted in the left axilla, these being excised at this time, coincident with bilateral salpingo-oophorectomy and hysterectomy. The hysterectomy had been performed for a fibroid tumor, and at operation metastatic mammary carcinoma was found in the left ovary.

Following the operative procedure the patient received deep x-ray therapy to the spine, lower back, left axilla, and supraclavicular area, in addition to testosterone propionate injections. She received 100 mg. of the hormone t.i.w., from April 22, 1947, to June 18, 1947, to a total of 3200 mg.

Despite the above therapy, new metastatic lesions were noted in the right ilium and right femur in July 1947. The patient then received additional injections of testosterone, 1000 mg. being given between July 5, 1947, and October 11, 1947. However, bone metastases continued to appear and enlarge, associated with pain in the areas involved.

On January 15, 1948, the patient was started on the oral administration of the glycolytic inhibitors according to the doses indicated above, and she also received 3 injections of 100 mg. of testosterone propionate, followed by an oral dose of 10 mg. of methyl testosterone per day. There was relief of pain and a definitely improved general status by January 26, 1948, which was maintained until the early part of March 1948, when some increase in metastatic bone involvement was noted. At this

time the glycolytic inhibitors were stopped and sodium azide started. However, by May 13, 1948, further progression of the bone metastases occurred, associated with pain in the shoulder and hip. Once again the glycolytic inhibitors were administered, but no significant effect was noted as the bone lesions increased, and skin nodules appeared in the area of the operative scar and grew rapidly. On November 4, 1948, intravenous glycolytic inhibitor therapy was started and continued until December 4, 1948, the average daily doses being sodium fluoride, 274.2 mg./day, iodoacetic acid, 101.3 mg./day, and malonic acid, 1804 mg./day. During this period the bone lesions remained essentially the same, with some questionable increase in sclerotic appearance, while the skin nodules showed some slight regression. Biopsy of these nodules after treatment showed no significant histological change which could be attributed to the treatment.

The patient felt well and was without complaints for several weeks after cessation of treatment when she began to have pain in the right femur. X-ray examination revealed enlargement of a previously existent metastatic bone defect, and the patient was readmitted to the hospital for radiation therapy to that area. Although the skin lesions had shown no evidence of increase in number or growth during and shortly after the intravenous therapy, after 1 month of no treatment increased growth of the old nodules and the appearance of new ones have been noted. This appears to be progressing at present (February 1949).

The apparent curtailment of growth of the skin nodules in association with enzyme inhibitor therapy and reactivation when treatment ceased is suggestive but certainly not conclusive of definite response to treatment. However, the occurrence of these suggestive changes in the Questionably Responsive group is a phenomenon which has not been observed in the group which we have termed Non-Responsive.

DISCUSSION

It would appear from the observations reported above that while certain types of cancer behave as if they were particularly dependent upon glycolytic mechanisms for their energy requirements, this is certainly not the case for all or even the majority of them. Further, even in those cases which may exhibit definite regression under glycolytic inhibitor therapy, an insensitivity develops and reactivation of the process occurs. In some cases this may be controlled by azide in accordance with the concept of preferential energy pathways discussed in our previous paper. Unfortunately, insensitivity to both systems of agents eventuates, and the growth of the tumor recurs. Whether or not more adequate therapeutic results would be obtained if the active agents functioned as prosthetic groups of larger organic molecules is worthy of investigation.

In a review of cancer therapy, Woglom (5) cited fluoride and iodoacetic acid as agents which might have deleterious effects on tumors by virtue of their ability to interfere with glycolysis. However, he dismissed them because of their toxicity. Our experience would indicate that the toxicity of these compounds has been overemphasized. It is true that sodium fluoride if taken by mouth will cause gastritis and regurgitation even in relatively small doses; however, when given in combination with an amphoteric antacid or as an enteric coated tablet so as to prevent the formation of hydrofluoric acid in the stomach, minimal complaints are encountered.

The relative lack of toxicity found in this series is in striking contrast with the effects of other chemotherapeutic agents, *viz.*, nitrogen mustards, urethane, and aminopterin. This would suggest that the mechanism of action was based on a cell function of only secondary importance to normal cells, but of significant moment to the malignant growths inhibited, and in this regard would be in line with the *in vitro* observations of the metabolism of cancer tissue. However, significant effects were attained only in a small number of malignant types, and those types include the ones (Hodgkin's disease and lymphosarcoma) most easily influenced by the other agents mentioned above and by radiation therapy. This limitation of effect is at variance with the similarity of metabolic characterization of diverse cancer tissues as disclosed by *in vitro* studies. Further studies are needed to clarify the basis of the special broad sensitivity of the lymphomas to a variety of chemical agents and radiation.

The variation in response of different cancer types might be a reflection of a difference in drug concentration delivered to the growth, a factor which would depend on such things as vascular supply, desmoplasia, etc. However, if these factors are equal, then certain questions must be raised as to the general applicability of the *in vitro* studies emphasizing the predominant role of glycolytic reactions in diverse types of tumor tissue.

In view of the uniformity of tumor tissue metabolism as studied *in vitro*, it would be expected that chemotherapeutic procedures that could inhibit a unique or essential property of one type of cancer should be able to produce similar changes in most if not all others. To date, however, the variety of

chemical agents possessing any selectivity of action on any neoplastic tissue as contrasted to its normal homologue have all been dismally ineffective in their action on diverse forms of cancer. That this is so is attested to not only by the results just cited but also by the clinical experience with agents like urethane, nitrogen mustards, aminopterin, Fowler's solution, hormone therapy, and radiation therapy itself.

The limitation of the therapeutic efficacy of these agents to individual tumors or related neoplastic types would seem to be extremely significant and cannot be underestimated in our concepts of malignancy, particularly in regard to attempts at the development of a rational chemotherapeutic control. At least two possible alternative explanations seem plausible. Either the inhibitory effect with the individual compounds represents an action on a unique metabolic or structural feature of these particular growths, or the concept of an essential entity of all malignant neoplasia as tacitly predicated on the basis of chemical and cytological observation is false or inadequate. That is, the *in vitro* biochemical characterization of tumor tissue may be a measure of the manifestations of malignancy rather than of its subtle essence, or it may fail to reflect the ability of tissues to adapt to unfavorable environmental factors by alterations of their metabolic pathways. The observation that even in the so-called Responsive group many cases failed to be influenced by these agents, or if initially affected, later became unresponsive, casts doubt on the essential role of accentuated glycolytic activity in tissue undergoing malignant transformation. Much remains to be learned of the mechanism and relative significance of the acquisition of biologically malignant potentialities and the development of chemical and morphological anaplasia.

SUMMARY

In a study of the effect of the glycolytic inhibitors fluoride, iodoacetate, and malonate on a series of more than 100 cases of diverse forms of human cancer, it was found that while objective evidence of tumor inhibition was obtained in cases of acute leukemia, Hodgkin's disease, and lymphosarcoma, this was not the case in patients with carcinoma of the colon, fundus uteri, ovary, pancreas, rectum, or in chronic leukemia, melanoma, chorionepithelioma testis, or with squamous cell cancers of the cheek and pharynx. Some responses of a less definitive nature were elicited in cancers of the breast, adrenal cortex, cervix, lung, stomach, and testes. These observations suggest that while certain cancer types behave as if they were particularly dependent on glycolytic mechanisms for their energy requirements, this is certainly not the case for all or even the majority of them. Further, even in those cases which may exhibit definite regression under glycolytic inhibitor therapy, an insensitivity develops and reactivation of the process occurs. These findings are discussed in relation to *in vitro* observations of cancer metabolism.

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